Novel Treatment of Breast and Prostate Cancer, and Mesothelioma by Targeting Cancer Stem Cells

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Abstract

Cancer is a major cause of death in the western world and is becoming an increasing world-wide problem. Even though treatment and therapeutic approaches to cancer have become better and there has been great improvement in the diagnosis of this group of pathologies, many cancer types are still lethal as there is no substantial treatment available. Further, even if treatment is successful, there is a significant risk of tumour recurrence, and such a tumour is then even harder to treat due to the acquired resistance in response to exposure to the initial treatment used. Cancer stem cells (CSCs) have been suggested as a reason behind more resistant tumours and tumour recurrence. Hence, CSCs are emerging as an important target for chemotherapy in order to suppress the re-initiation of tumours. The work of this laboratory has focused on two mitocans (mitochondrially-targeted anti-cancer compounds), α-tocopheryl succinate and its mitochondrially targeted derivative MitoVES. These anti-cancer agents have been shown to be effective against a variety of cancer cells and several in vivo mouse models of cancer, while being non-toxic to normal tissue. Whether they are able to target CSCs was one of the main aims of this study. This thesis addresses several questions in regards to CSCs. Firstly, breast and prostate cancer cell lines (MCF7 and LNCaP, respectively) and a mesothelioma cell line (IstMes2) as well as primary glioblastoma cells grown in the form of spheres were confirmed as a valid model to study CSC biology and resistance to apoptosis, as both phenotypical and genotypical features confirmed increased levels of stemness. It was shown that these cell lines grown as spheroids in culture exerted increased resistance to apoptosis due to the increased levels of FLIP and upregulated indoleamine 2,3-dioxygenase (IDO), which could endow them with increased propensity to evade the immune system. Furthermore, it was demonstrated that mitocans, especially MitoVES, are able to overcome this ‘evasive’ nature of CSCs by lowering the increased levels of IDO and that these agents were also able to efficiently trigger apoptosis in the populations containing increased stemness. It was also demonstrated that MitoVES exerted its effect through functional mitochondrial complex II. In conclusion, it is proposed that a highly efficient way to eradicate tumour cells, including both fast-proliferating cancer cells and otherwise drug-resistant CSCs, could be achieved by using anti-cancer agents like MitoVES that would kill the bulk of the tumour cells, as well as inhibit IDO. The net outcome would be to allow for the cells of the immune system to attack the remaining tumour cells. It is also speculated that
combining MitoVES with other IDO inhibitors might be even more effective for eliminating tumours. Due to high resistance of the cells to current therapies and tumour recurrence, it is proposed that this work has considerable translational potential.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where referenced.

Signed:


________________________________________
Renata Zobalová

December 2012
Acknowledgement of published papers included in this thesis

Included in this thesis are 4 published papers in Chapters 3, 4, 5 and 6 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details for these papers are:


Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each published paper.

(Signed) __________________________________________
Renata Zobalová

(Countersigned) __________________________________
Supervisor: Associate Professor Stephen J. Ralph
Acknowledgements

Firstly, I would like to thank Associate Professor Stephen J. Ralph for his supervision, support, guidance and optimism throughout this research project. I would also like to thank very much Professor Jiri Neuzil for his assistance, help, great ideas, support and enthusiasm for research, which worked as great motivation. Many thanks go to everyone in the laboratory, Dr. Lan-Feng Dong, Maria Nguyen, Jacob Goodwin and Elham Alizadeh Pasdar for helping me with a variety of techniques and being supportive and encouraging. Also, I would like to thank Michael Stapelberg for his help with the microarray analysis and many other experiments without which this work would not be possible and also for being a good friend, always very optimistic. My special thanks go to Marina Stantic, my great friend, with whom we struggled together and helped each other to overcome the obstacles of our PhD studies.

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<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ALDH1</td>
<td>Aldehyde dehydrogenase 1</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukaemia</td>
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<tr>
<td>ANT</td>
<td>Adenine nucleotide translocase</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<td>APL</td>
<td>Acute promyelocytic leukaemia</td>
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<tr>
<td>ATO</td>
<td>Arsenic trioxide</td>
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<td>ATRA</td>
<td>All-trans-retinoic acid</td>
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<td>α-TOH</td>
<td>α-Tocopherol</td>
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<tr>
<td>α-TOS</td>
<td>α-Tocopheryl succinate</td>
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<td>BCR-ABL</td>
<td>Breakpoint cluster region-Abelson</td>
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<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
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<tr>
<td>Bmi-1</td>
<td>B lymphoma Mo-MLV insertion region 1</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>3BP</td>
<td>3-Bromopyruvate</td>
</tr>
<tr>
<td>CamKII</td>
<td>Calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CBF</td>
<td>C promoter binding factor</td>
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<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
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<td>CIDE</td>
<td>Cell death-inducing DFF45-like effector</td>
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<td>CML</td>
<td>Chronic myelogenous leukaemia</td>
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<td>CSC/TIC</td>
<td>Cancer stem cells/Tumour-initiating cells</td>
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<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<td>CII</td>
<td>Mitochondrial complex II</td>
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<td>DCA</td>
<td>Dichloroacetate</td>
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<td>DCC</td>
<td>Deleted in colorectal carcinoma</td>
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<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
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<td>DeR</td>
<td>Decoy receptor</td>
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<tr>
<td>DD</td>
<td>Death domain</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>------</td>
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<tr>
<td>ΔΨm</td>
<td>Mitochondrial inner trans-membrane potential</td>
</tr>
<tr>
<td>2DG</td>
<td>2-Deoxyglucose</td>
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<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
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<td>DISC</td>
<td>Death-inducing signalling complex</td>
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<td>DR</td>
<td>Death receptor</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>ENDOG</td>
<td>Endonuclease G</td>
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<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
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<td>EPP</td>
<td>Extrapleural pneumonectomy</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<td>ErbB-2</td>
<td>Erythroblastic leukaemia viral oncogene homolog 2</td>
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<tr>
<td>ESA</td>
<td>Epithelial specific antigen</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<td>FADD</td>
<td>Fas-associated death domain</td>
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<td>FLIP</td>
<td>Flice-like inhibitory protein</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<td>GSI-18</td>
<td>[11-endo]-N-(5,6,7,8,9,10-hexahydro-6,9-methanobenza[a][8]annulen-11-yl)-thiophene-2-sulfonamide</td>
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<td>GSK-3</td>
<td>Glycogen synthase-3</td>
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<td>HAT</td>
<td>Histone acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>HH</td>
<td>Hedgehog</td>
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<td>HIF-1α</td>
<td>Hypoxia inducible factor-1 alpha</td>
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<td>HMG</td>
<td>High mobility group</td>
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<tr>
<td>HPV</td>
<td>Human papilloma viruses</td>
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<tr>
<td>HTRA2</td>
<td>High temperature requirement protein 2</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
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<tr>
<td>IDC</td>
<td>Infiltrating (or invasive) ductal carcinoma</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN(γ)</td>
<td>Interferon (γ)</td>
</tr>
<tr>
<td>IKZF1</td>
<td>Ikaros family zinc finger 1</td>
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<td>Neighbor</td>
<td>Term</td>
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<td>ILC</td>
<td>Infiltrating (or invasive) lobular carcinoma</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity-modulated radiotherapy</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
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<tr>
<td>LEF/TCF</td>
<td>Lymphoid enhancer factor/T cell receptor</td>
</tr>
<tr>
<td>LRP5/6</td>
<td>Low-density lipoprotein receptor-related proteins 5 and 6</td>
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<tr>
<td>MELK</td>
<td>Maternal embryonic leucine zipper kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>MIM</td>
<td>Mitochondrial inner membrane</td>
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<tr>
<td>MitoVES</td>
<td>Mitochondrially targeted vitamin E succinate</td>
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<td>MMTV</td>
<td>Mouse mammary tumour virus</td>
</tr>
<tr>
<td>MOM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>Msi1</td>
<td>Musashi-1</td>
</tr>
<tr>
<td>1MT</td>
<td>1-Methyl tryptophan</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic/severe combined immunodeficiency</td>
</tr>
<tr>
<td>PCG</td>
<td>Polycomb group</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PIA</td>
<td>Proliferative inflammatory atrophy</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PML-RAR α</td>
<td>Promyelocytic leukaemia-retinoic acid receptor alpha</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
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<td>rhEGF</td>
<td>Recombinant human epidermal growth factor</td>
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<tr>
<td>rhFGF</td>
<td>Recombinant human fibroblast growth factor</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SFRPs</td>
<td>Secreted Frizzled-related proteins</td>
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<td>SMase</td>
<td>Sphingomyelinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
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<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
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<td>TDO</td>
<td>Tryptophan dioxygenase</td>
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<td>TGF-α</td>
<td>Transforming growth factor-alpha</td>
</tr>
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<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>TILs</td>
<td>Tumour-infiltrating lymphocytes</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour/nodes/metastasis</td>
</tr>
<tr>
<td>TPP⁺</td>
<td>Triphenylphosphonium</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related-apoptosis-inducing-ligand</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>T-regulatory</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Wnt-1</td>
<td>Wingless-type MMTV integration site family, member 1</td>
</tr>
</tbody>
</table>
List of Publications


1 Introduction

1.1 Aims and Objectives

Cancer stem cells (CSCs) are emerging as an important target for chemotherapy in order to suppress the renewal of tumours.

Breast cancer is one of the most frequently occurring cancers among females world-wide, and it is the most common cancer among women in Australia with currently one in nine women expected to be diagnosed with breast cancer during their life-span (Cancer Council Australia, 2008). Prostate cancer is one of the most frequently occurring cancers in men world-wide and it is the most common cancer in Australian men (after non-melanoma skin cancer), with one in four males expected to be diagnosed with prostate cancer during their life-span, of which approximately one sixth will die from the disease (Cancer Council Australia, 2008). Mesothelioma is, on the other hand, a relatively rare form of cancer, which affects both males and females. However, it has a very high mortality rate and very limited treatment options. In Australia, the annual incidence rate of new cases is around 30 per million (Leigh et al., 2002; safeworkaustralia.gov.au, 2012), of which approximately 80% of the people diagnosed with this disease will die within a year.

Even though treatment has improved in recent years to prolong the life of patients, secondary tumours often form after the initial therapy and recovery, leading to the relapse of the disease (Neuzil et al., 2007). Recently it has been suggested that there is a small population of tumour cells that have stem-like characteristics. These ‘cancer stem cells’ are similar to somatic stem cells, capable of self-renewal, asymmetric division and multilineage differentiation (D’Amour and Gage, 2002; Singh et al., 2004; Lou and Dean, 2007). They have been identified in various malignancies, such as leukaemias, myelomas, brain and breast carcinomas, as well as in tissues prone to malignant conversion (Park et al., 1971; Lapidot et al., 1994; Bhatia et al., 1998; Lou and Dean, 2007). CSCs express high levels of the ATP-binding cassette (ABC) drug transporters, exhibit higher activity of DNA repair systems, are less likely to enter apoptosis and have the ability to evade immune surveillance (Singh et al., 2004; Wicha et al., 2006; Lou and Dean, 2007; Tang et al., 2007; Hanahan and Weinberg, 2011). These features render them relatively resistant to established therapeutic means, including chemotherapy and radiation, for which reasons they present a considerable problem in cancer management.
A variety of surface markers have been used to characterise CSCs. Different studies have shown that these cells have elevated expression of the stem cell surface marker CD133 (Richardson et al., 2004; Mehra et al., 2006). CD133 is a cell surface glycoprotein comprising five trans-membrane domains and two large glycosylated extracellular loops (Miraglia et al., 1997; Corbeil et al., 2001). Its function has not been established thus far. Nevertheless, it can be used to distinguish between more differentiated cancer cells and CSCs. It is possible to maintain CD133+ cells in culture and use them for tumour generation in immunocompromised mice (Suetsugu et al., 2006; Yin et al., 2007), indicating that CD133 is a true cancer stem cell marker.

Another marker of interest is the CD44 surface protein. Overexpression of CD44 has been found in different CSCs in breast, prostate and pancreatic cancers (Al-Hajj et al., 2003; Collins et al., 2005; Li et al., 2007). The CD44 protein is involved in cell-cell and cell-matrix interactions, and CD44+ breast cancer cells exert more pronounced transforming growth factor β (TGF-β) signalling (Shipitsin et al., 2007).

Apoptosis operates via two major pathways, the intrinsic or mitochondrial-mediated pathway and the extrinsic or receptor-mediated pathway. Cells that are damaged or not ‘normal’ go into apoptosis thereby removing them and helping to maintain functional organisms/tissues. On the other hand, within the context of malignant pathophysiology (such as neoplastic diseases), cancer cells can be forced to commit ‘suicide’ using a variety of agents, including the vitamin E analogue epitomised by α-tocopheryl succinate (α-TOS). By contrast, CSCs do not readily undergo apoptosis due to resistance mechanisms, although the effect of α-TOS on such cells has not previously been tested. The vitamin E analogue triggers apoptosis by increasing intracellular reactive oxygen species (ROS) production/accumulation, which induces the mitochondrial-mediated apoptotic pathway. α-TOS and its analogues have recently been modified to provide greater targeting to cancer cell mitochondria. This class of drugs belongs to the recently defined group of selective anti-cancer compounds termed ‘mitocans’ (Neuzil et al., 2007).

Mitocans have proven to be very efficient at inducing apoptosis in breast cancer cells. Furthermore, preliminary unpublished results indicate that treatment of breast CSCs with mitocans also induces apoptosis in these cells. Therefore, there is a great potential for these agents as they may overcome CSC resistance by being targeted directly to mitochondria, unlike many established chemotherapeutics. Hence, studying
the resistance of CSCs to treatment using these novel anti-cancer agents may be of significant clinical relevance.

The main aim of this project was to overcome the resistance of breast, prostate and mesothelioma CSCs to treatment and to develop efficient therapeutic strategies that would potentially help eradicate these resistant cells and prevent tumour recurrence. More precisely, the objectives of this study were to establish the level of responsiveness of CSCs to the anti-cancer drugs from the group of vitamin E analogues and, in particular, determine the molecular mechanisms behind the CSC resistance to anti-cancer drugs and, more specifically, to determine what makes these cells resistant to treatment in comparison with ‘normal’ cancer cells. The link between the resistance, the immune system and apoptosis was also studied in these relatively resistant CSCs in order to develop therapeutic strategies, based on the use of vitamin E analogues that would overcome the immune system resistance and promote killing of CSCs.

1.2 Significance

Cancer is a major cause of death in industrialised countries and is becoming an increasing problem world-wide. It is a pathology whose management is highly challenging, and no effective treatment currently exists for several types of cancer. Further, even though some types of neoplasias can be treated, the possibility of the disease returning remains significant. Recent research has suggested that re-occurrence of cancer is caused largely by CSCs. These cells share similarities with normal stem cells and hence have the propensity to differentiate into rapidly proliferating cancer cells. These cells do not readily, if at all, respond to the current treatments. In view of this problem, this project is highly significant as a major focus of this study was to determine the molecular mechanisms underlying resistance of CSCs to therapy. A second aim was to identify an efficient anti-cancer agent that will target and kill CSCs, based on vitamin E analogues. Developing an anti-cancer agent that would ensure very little or no cancer recurrence would represent a significant break-through in cancer biology.
2 Literature Review

2.1 Introduction: Definition and Types of Cancer

Cancer is a major cause of death in the western world and it is becoming an increasing world-wide problem. It is a disease that is very difficult to treat. While for certain types of cancer there is no effective treatment presently available, other pathologies can be treated but the possibility of relapse remains significant. In the simplest form, cancer can be defined as a pathological condition characterised by uncontrolled growth, spread and invasion of abnormal cells (American Cancer Society, 2008). If this spread is not controlled, it can result in death. Cancer is not a single disease; rather, it is a group of more than 100 different and distinctive pathologies. It can evolve in practically any tissue of the body and has many different forms, depending on its location and the tissue/cell of origin.

The majority of human cancers arise in the epithelium (the layer of cells covering the surface of the body and the lining of internal organs and various glands). These types of neoplastic diseases are referred to as carcinomas. Sarcomas are malignancies of the connective tissue of the body, such as bones, muscles and blood vessels. Cancers arising from haematopoietic cells and cells of the immune system include leukaemias and lymphomas. Gliomas are cancers of the nerve tissue, while melanomas arise from darkly pigmented melanocytes, usually in the skin (American Cancer Society, 2008).

The frequency of a particular cancer may depend on a person’s gender. While skin cancer is the most common type of malignancy for both men and women, the second most common type in women is breast cancer and for men it is prostate cancer. The frequency of cancer does not equate to its mortality. For example, non-melanoma skin cancers are relatively frequently curable, whereas lung cancer is the leading cause of death from cancer for both men and women in the United States (American Cancer Society, 2012).

2.1.1 Breast Cancer

Breast cancer is one of the most frequently occurring cancers in women world-wide and it is the most common cancer in Australian women, with one in nine females predicted to be diagnosed with breast cancer during their life-span, of which approximately one fifth will die from the disease (Cancer Council Australia, 2008).
The main parts of the female breast are lobules (milk-producing glands), ducts (milk passages that connect the lobules and the nipple), and stroma (fatty tissue and ligaments surrounding the ducts and lobules, blood vessels, and lymphatic vessels) (Figure 1).

*Figure 1: Structure of the normal breast* (Westmead Breast Cancer Institute, 2010).

During its lifetime, the mammary gland undergoes many stages of development and differentiation. Most development occurs during puberty, when the ductal epithelium expands by branching and invading the surrounding fat pad to form an organised mammary tree. During its existence, the epithelium will go through a number of cycles of proliferation and cell death as a result of pregnancy, lactation and involution (Lanigan *et al.*, 2007). Many of the signalling mechanisms which control the initial invasion of the fat pad by the epithelium and its continuing plasticity can be exploited or ‘hijacked’ by tumour cells in order to support their aberrant growth and progression towards the invasive phenotype. This is not only true for epithelial cells, but it also applies to the surrounding microenvironment, including fibroblasts, macrophages and adipocytes (Lanigan *et al.*, 2007). This means that breast carcinomas have heterogeneous pathologies and molecular profiles, and, in some ways, the diversity of breast cancers resembles haematological malignancies more than other epithelial cancers (Stingl and Caldas, 2007), although cancers are now considered to be a
heterogeneous population, not a homogeneous one as thought previously (Hanahan and Weinberg, 2011; Gerlinger et al., 2012).

Several different types of breast cancer have been classified and the following definitions are derived from the Westmead Breast Cancer Institute, 2010. Nearly all breast cancers start in the ducts or lobules of the breast. Due to the fact that breast is glandular tissue, these malignancies are called adenocarcinomas. The two main types of breast adenocarcinomas are ductal and lobular carcinomas. In situ means that the cancer stays restricted to ducts or lobules and has not invaded surrounding fatty tissues in the breast or spread to other organs in the body. There are two types of breast carcinoma in situ, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). Lobular carcinoma in situ, also called lobular neoplasia, starts in the lobules, but does not penetrate through the lobule walls. Most breast cancer specialists propose that LCIS itself does not usually become invasive, but women with this condition do have a higher risk of developing invasive cancer in either breast in the future. Ductal carcinoma in situ is the most common type of non-invasive breast cancer (Figure 2). Cancer cells stay inside the ducts and do not spread through the walls of the ducts into the surrounding breast tissue.
Types of Breast Cancer:

Infiltrating (or invasive) ductal carcinoma (IDC) first develops in a milk passage, or duct, of the breast, before breaking through the wall of the duct and invading the surrounding fatty tissue. It can metastasise or spread to other parts of the body through the lymphatic system and through the bloodstream. The process of invasion and metastasis is shown in Figure 3. Infiltrating or invasive ductal carcinoma accounts for about 80% of all breast cancers.

Infiltrating (or invasive) lobular carcinoma (ILC) starts in the milk-producing glands. It can also metastasise to other parts of the body. About 10-15% of invasive breast cancers are represented by invasive lobular carcinomas.
Medullary carcinoma is a type of invasive breast cancer that has a relatively well-defined distinct boundary between tumour tissue and normal breast tissue. The cancer cells are bigger and there are immune cells present at the edges of the tumour. Prognosis for this type of carcinoma is better than that for invasive lobular or invasive ductal cancers. It accounts for about 5% of all breast cancers.

Colloid carcinoma, also called mucinous carcinoma, is a rare type of invasive disease formed by mucus-producing cancer cells. Prognosis for colloid carcinoma is also better than for invasive lobular or invasive ductal cancers.

Tubular carcinoma is a well-differentiated form of breast cancer characterised by invasion of the stroma by small epithelial tubules. This type of breast carcinoma accounts for about 2% of all breast cancers, and also has better prognosis than invasive ductal or lobular carcinomas.

Inflammatory breast cancer is a disease that causes inflammation and reddening of the skin of the breast. This is actually caused by cancer cells blocking lymph vessels or channels in the skin. This cancer accounts for 1–3% of all breast cancers.

Phyllodes tumour is a very rare form of breast cancer, which develops in the stroma of the breast. These tumours are usually benign.

Paget’s disease of the nipple forms firstly in the breast ducts before spreading to the skin of the nipple and then to the areola, which is the dark circle around the nipple.

Adenoid cystic carcinoma is a type of cancer that rarely develops in the breast; and is more frequently restricted to the salivary glands. Adenoid cystic carcinomas of the breast also have a better prognosis than invasive lobular or ductal carcinomas.
Figure 3: A model of breast carcinoma progression. Schematic view of normal, *in situ*, invasive, and metastatic tumour progression (Polyak, 2007).

Histological classification of breast cancer is based on the presence or absence of abnormal cells originating within the breast. A sample of breast cells may be taken following breast biopsy, lumpectomy or mastectomy. Pathologists focus on three major features when determining the grade of the carcinoma: tubule formation (percentage of carcinoma composed of tubular structures), nuclear pleomorphism (changes in cell size and uniformity) and frequency of cell mitosis (the rate of cell division). Each of these features is given a score ranging from 1-3 (1 indicating slower cell growth and 3 indicating faster cell growth). The scores are then added to give the final score that ranges between 3 to 9 (Table 1) (American Medical Association, 2008).
The Nottingham (also called Elston-Ellis) modification of the Scarff-Bloom-Richardson system is the most common type of cancer grade system used today (Table 2). The scores for tubule, nuclear pleomorphism and frequency of cell mitosis are added as described in the previous paragraph to give a final score between 3-9. A tumour with a final sum of 3-5 is considered a Grade 1 tumour (well-differentiated). A sum of 6 or 7 is considered a Grade 2 tumour (moderately-differentiated), and a sum of 8 or 9 is a Grade 3 tumour (poorly-differentiated) (American Medical Association, 2008).
Table 2: Scarff-Bloom-Richardson grade system for classifying cancer stage
(American Medical Association, 2008).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
<th>Score</th>
<th>5 year survival</th>
<th>7 year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>Well-differentiated breast cells; cells generally appear normal and are not growing rapidly; carcinomas arranged in small tubules.</td>
<td>3, 4, 5</td>
<td>95%</td>
<td>90%</td>
</tr>
<tr>
<td>Grade 2</td>
<td>Moderately-differentiated breast cells; have characteristics between Grade 1 and Grade 3 tumours.</td>
<td>6, 7</td>
<td>75%</td>
<td>63%</td>
</tr>
<tr>
<td>Grade 3</td>
<td>Poorly differentiated breast cells; cells do not appear normal and tend to grow and spread more aggressively.</td>
<td>8, 9</td>
<td>50%</td>
<td>45%</td>
</tr>
</tbody>
</table>

Molecular classification of breast cancers, which is now commonly used, is based on gene expression profiling studies and it categorises breast cancer into three major subtypes, the luminal, basal-like, and HER2+ (Erb-B2+) breast cancers. They are further characterised by different transcriptomic features and are predictive of responses to specific therapies (Perou et al., 2000; Sørlie T et al., 2001; Rakha et al., 2010). The subtypes also seem to have a consistent gene expression between primary tumours and subsequent metastatic lesions, which may occur with great latency (Weigelt et al., 2005), and they are also associated based on differences in the clinical outcome (Perou et al., 2000; Sørlie T et al., 2001). Further studies expanded the molecular classification to distinguish between luminal A and luminal B breast cancer (Sørlie T et al., 2001; Yu et al., 2004). More studies are being performed in order to expand the molecular classification system, make it more detailed and take into account various mutations such as the BRCA1 and BRCA2 mutations (Larsen et al., 2013).

The TNM Staging System (abbreviated from Tumour/Nodes/Metastases) is one of the most commonly used staging systems for different cancer types, including breast cancer. This system was developed and is maintained by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). It was
developed as a tool for doctors to stage different types of cancer based on certain standard criteria (Appendix 1). Each cancer type has its own specific classification system based on the TNM staging system, which means that the letters and numbers do not always have to refer to an identical item (American Joint Committee on Cancer, 2010).

2.1.2 Prostate cancer
Prostate cancer is one of the most frequently occurring cancers in men world-wide and it is the most common cancer in Australian men (after non-melanoma skin cancer), with one in four males expected to be diagnosed with prostate cancer during their life-span, of which approximately one sixth are likely to die from the disease (Cancer Council Australia, 2008).

The prostate is a part of the male reproductive system that helps make and store seminal fluid. In adult men, a typical prostate is about three centimetres long and weighs about twenty grams (Aumüller, 1979) (Figure 4). It is located in the pelvis, under the urinary bladder, in front of the rectum. The prostate surrounds a part of the urethra, which is the tube that carries urine from the bladder during urination and semen during ejaculation (Moore and Dalley, 1999). Due to its location, prostate pathologies often affect urination, ejaculation, and quite rarely defecation, as it gets enlarged and constricts the urethra (Figure 5). The prostate contains many small glands which make about twenty per cent of the fluid constituting semen (Aumüller, 1979; emedicinehealth, 2012).
The prostate gland starts to develop before birth. It grows rapidly during puberty, driven by male hormones, androgens. The main androgen, testosterone, is made in the testicles. Testosterone is converted into dihydrotestosterone (DHT) by the
enzyme 5α reductase. DHT is the main hormone, which signals to the prostate to grow. The prostate usually stays at about the same size or grows slowly in adults, as long as male hormones are present (American Cancer Society, 2012).

There are four main disorders of the prostate and they include: prostatitis, benign prostatic hyperplasia (BPH), prostatodynia and prostate cancer (Prostate Cancer Foundation of Australia, 2012).

Prostate cancer is one of these four disorders, and is potentially life-threatening. One of the major problems is that these conditions exert similar symptoms and an even bigger problem is that most prostate cancers develop without any symptoms present. There are several types of cells found in the prostate, however nearly all prostate cancers develop from the gland cells. This type of cancer is called adenocarcinoma. There are other types of cancer, which can also start in the prostate gland, such as sarcomas, small cell carcinomas and transitional cell carcinomas. These types of prostate cancer are very rare, most prostate cancers are adenocarcinomas. Some prostate cancers can have quick progression and result in metastasis. However, most prostate cancers are slow growing and male patients die of other causes first, without prostate cancer affecting them or even without clearly diagnosed prostate cancer.

There are also several pre-cancerous conditions of the prostate, which may lead to prostate cancer. These include: prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) (American Cancer Society, 2012).

Prostatic intraepithelial neoplasia (PIN) is a condition, where the prostate gland cells become abnormal, however they do not metastasise. PIN is sub-divided into two types based on the patterns of cell abnormality:

- Low-grade PIN: the pattern of prostate cells appears almost normal.
- High-grade PIN: the pattern of prostate cells appears more abnormal.

PIN is a very common condition. It can occur already in the early twenties in men and nearly half of all men have this condition by the time they are fifty. Many men have low-grade PIN and do not go on to develop prostate cancer. However, 20-30% of men with high-grade PIN tend to progress into cancer in another area of the prostate.

Proliferative inflammatory atrophy (PIA) is a condition in which prostate cells appear smaller, and it is accompanied by inflammation in the area. Researchers believe that PIA may sometimes lead to high-grade PIN or even directly to prostate cancer.

Prostate cancers are most commonly graded using the Gleason Grading System, which is based on the histological characteristics of the glandular architecture found
within the tumour (American Cancer Society, 2012). This system involves the cellular content and tissue architecture from biopsies and provides an estimate of the destructive potential and ultimate prognosis of the disease. The Gleason System assigns a Gleason grade, using numbers from 1 to 5 depending on the appearance of the cancerous tissue in comparison to normal prostate tissue (Figure 6) (Prostate Cancer Research Institute, 2012):

If the cancerous tissue looks very similar to normal prostate tissue, the grade of 1 is assigned.

If the cancer cells and their growth pattern look very abnormal, a grade of 5 is assigned and the tissue is referred to as the grade 5 tumour.

Grades 2 to 4 have features between these extremes.

Due to the fact that prostate cancers quite regularly feature areas with different grades, a grade is allocated to the two areas that make up most of the tumour. These two grades are added together to yield the Gleason score (also called the Gleason sum), which is between 2 and 10 (Table 3). Most prostate biopsies are grade 3 or higher, grades 1 and 2 are used very rarely (American Cancer Society, 2012).
Figure 6: The classical Gleason grading system diagram showing five basic tissue patterns with assigned tumour grades (Prostate Cancer Research Institute, 2012).

Table 3: The Gleason score based on the Gleason Grading System (American Cancer Society, 2012).

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Cancer grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 or less</td>
<td>low-grade</td>
<td>well-differentiated cells</td>
</tr>
<tr>
<td>7</td>
<td>intermediate-grade</td>
<td>moderately differentiated cells</td>
</tr>
<tr>
<td>8 to 10</td>
<td>high-grade</td>
<td>poorly differentiated cells</td>
</tr>
</tbody>
</table>

The higher the Gleason score, the more likely it is that the cancer will grow and spread quickly.

The Gleason score is the best classification of prostate cancer at the moment (Markert et al., 2011). Unlike for other cancer types such as breast or colon carcinomas, there is no molecular classification system for prostate cancer (Getzenberg, 2010). There have been several studies, which tried to classify prostate cancer on the molecular level, however, this type of classification is not used at the moment in the clinical settings. For example a study by Markert et al. classified prostate cancer using mRNA
microarray signature profiles, which differentiated prostate cancer into five subtypes based on the embryonic stem cell expression pattern (stemness), inactivation of the tumour suppressors p53 and PTEN, activation of several oncogenic pathways, and the TMPRSS2–ERG fusion (Markert et al., 2011). Another study by Taylor et al. examined global DNA copy number together with expression profiles in order to distinguish the types of the disease as well as to provide novel treatment targets (Taylor et al., 2010). A different approach for molecular classification was to use chromatin immunoprecipitation (ChIP) together with extensive sequencing in order to get an understanding of the genetic pathways associated with progression of prostate cancer (Yu et al., 2010). Hence, progression in establishing a molecular classification system of prostate cancer that would help with prediction of response to treatment and clinical outcome is being made.

An important aspect of evaluating prostate cancer is determining the stage of the cancer as well as how far the tumour tissue has spread. This helps in defining the prognosis and it is very useful when selecting therapies. The most common system is the four-stage TNM system (Appendix 1). Its constituents include the size of the tumour, the number of involved lymph nodes, and the presence of any other metastases (American Joint Committee on Cancer, 2009). The TNM staging system was discussed in the previous chapter.

2.1.3 Mesothelioma

Mesothelioma is a rare form of cancer, which has a very high mortality rate with quite limited therapeutic options. The incidence of mesothelioma varies greatly depending on the country. In Australia the annual incidence rate of new cases is around 30 per million (Leigh et al., 2002; safeworkaustralia.gov.au, 2012), of which approximately 80% of the people diagnosed with this disease will die within 12 months. Mesothelioma occurs in both males and females. However, it is much more predominant in males with 80-90% of new cases (safeworkaustralia.gov.au, 2012).

The etiology of malignant mesothelioma is mainly associated with exposure to asbestos fibres. There are two forms of asbestos, the rod-shaped amphiboles, which are more carcinogenic, and the curly chrysolite form, which is less carcinogenic (Rees et al., 1999; Hodgson and Darnton, 2000). Mesothelioma occurrence has also been associated with another mineral fibre, erionite (Baris et al., 1978; Artvinli et al., 1979) and irradiation (Antman et al., 1983; Gilks et al., 1988; Small et al., 2008; Goodman et
Several studies have also suggested that Simian Virus 40 (SV40) has a possible carcinogenic or co-carcinogenic role in initiation of mesothelioma (Cicala et al., 1993). Development of mesothelioma depends on the duration and intensity of exposure, although even very short or very mild exposures to asbestos may be enough to cause tumour formation. Mesothelioma has long latency periods from first exposure to diagnosis, ranging from 14 to 72 years, depending on the extent and type of exposure (Bianchi et al., 1997). There are two main ways in which the fibres enter the body: either through the mouth and nose by inhalation or through the mouth by eating and drinking (Figure 7) (Mesothelioma Information Resource Group, 2009).
Figure 7: Two ways in which asbestos fibres enter the body and the outcomes relative to the way of admission (Mesothelioma Information Resource Group, 2009).

Mesothelioma can be classified into three types based on tumour location or histological type (safeworkaustralia.gov.au, 2012; Jongsma et al., 2008):
Pleural mesothelioma arises in the pleura, the thin serous lining encapsulating the lungs and lining the walls of the thoracic cavity (Figure 8). This type of mesothelioma is the most common, with 94% of all cases.

Peritoneal mesothelioma arises in the peritoneum, which is a thin serous membrane that covers the abdominal organs and lines the walls of the abdominal and pelvic cavities (Figure 9). Peritoneal mesothelioma accounts for about 5% of the cases.

Pericardial mesothelioma is the least common. It forms in the pericardium, which is a fibroserous sac enclosing the heart and the roots of the great vessels, consisting of two layers: the visceral layer or epicardium and the outer parietal layer, which is lined with a serous membrane (Figure 10).

Mesothelioma can also be subdivided into three histological types. These are epithelial, sarcomatoid and mixed types. Sarcomatoid mesothelioma cannot be quite often easily discriminated from other sarcomas that can develop in the thoracic cavity such as leiomyosarcomas, rhabdomyosarcomas and osteosarcomas (Cagle et al., 1989; Attanoos et al., 2000). Unlike for other cancer types such as breast or colon cancer, there is no molecular classification system for mesothelioma. However, one of the criterias of mesothelioma, which indicates the prognosis, is the SV40 status, i.e. whether the cancer is SV40-positive or -negative, since SV40 inhibits the p53 activity.

Figure 8: Picture of pleural mesothelioma (mesothelioma.com, 2011).
Figure 9: Picture of peritoneal mesothelioma (mesothelioma.com, 2011).

Figure 10: Picture of pericardial mesothelioma (mesothelioma.com, 2011).
For classification of the different types and subtypes of mesothelioma and for the diagnosis, histological specimens are needed to assess tumour morphology (Carter and Otis, 1988; Cagle et al., 1989; Ordonez and Tornos, 1997; Khalidi et al., 2000). Traditional diagnostic techniques include pleural fluid cytology, which is obtained through thoracentesis, needle biopsy of pleural tissue using CT guidance, video-assisted thoracoscopy surgery with direct visualisation and biopsy of pleural nodules, and open thoracotomy, as well as oesophageal ultrasound (EUS), mediastinoscopy, and laparoscopy. The last three techniques are used more readily for the disease staging purposes (Mott, 2012).

There are several staging systems, which have been used for mesothelioma. Most of the staging systems deal with primary pleural mesothelioma, while peritoneal mesothelioma does not have its own staging system. The first staging system, which has been used for pleural mesothelioma, is the Butchart system. It is based on a simple description of the extent of the disease and disregards its histological subtype: Stage I refers to pleural contained tumour, Stage II chest wall or mediastinal invasion, Stage III peritoneal or diaphragmatic penetration and Stage IV distant metastases. Another staging system used is the Brigham system, which defines surgical resectability and lymph node involvement, although this system is no longer in common use. The most recent and readily used system is the TNM system, which was developed and modified for mesothelioma by the International Mesothelioma Interest Group (Table 4) (Mott FE, 2012). This staging system is also used for other cancers as was discussed in the previous chapters (Appendix 1).
Table 4: Tumour, node and metastasis staging for mesothelioma as defined by the International Mesothelioma Interest Group (adapted from Edge et al., 2010 and Mott, 2012).

<table>
<thead>
<tr>
<th>Stage</th>
<th>T1a-b, N0, M0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Ipsilateral parietal pleura, without or with the involvement of visceral pleura</td>
</tr>
<tr>
<td>Stage II</td>
<td>T2, N0, M0</td>
</tr>
<tr>
<td></td>
<td>Involving each ipsilateral pleural surface (parietal, mediastinal, diaphragmatic, visceral) with ≥ 1 of following: involving diaphragm muscle or extension into lung parenchyma</td>
</tr>
<tr>
<td>Stage III</td>
<td>T1-2, N1-2, M0 or T3, N0-3, M0</td>
</tr>
<tr>
<td></td>
<td>Above (T1-2) with ≥ 1 of following (T3): involve thoracic fascia, mediastinal fat solitary focus into chest wall, pericardium. Ipsilateral pulmonary/hilar nodes (N1), ipsilateral mediastinal or sub-carinal nodes (N2)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>T4, any N, M0 or any T, N3, M0 or any T, any N, M1</td>
</tr>
<tr>
<td></td>
<td>Tumour extension into CW with or without rib destruction, transdiaphragmatic extension into peritoneum, contralateral pleura extension, mediastinal organs, spine, into myocardium (T4). Contralateral mediastinal, or any supraclavicular nodes (N3) or distant metastases (M1)</td>
</tr>
</tbody>
</table>

Stage I, II, III – potentially resectable
Stage IV – unresectable

2.2 Therapeutic Approaches to Cancer
Cancer can be treated using several different therapeutic approaches. The classical pillars of cancer therapy include surgery, chemotherapy and radiation therapy (Morrison et al., 2011). Other treatments used at present more or less readily are hormone therapy and biological and targeted therapies such as active and adoptive immunotherapy, small-molecule inhibitors, monoclonal antibodies and tumour vaccines, which serve as prevention against particular cancers, for example the vaccine against the human papilloma viruses (HPV), which are responsible for over 70% of cervical cancers (Cassidy et al., 2010). An innovative and attractive approach to cancer therapy is personalised cancer treatment. Recent breakthroughs in research have allowed the
characterisation of malignancies according to their unique gene expression. This has allowed more accurate and realistic targeting of many cancer types depending on their specific gene expression patterns. Examples of this approach include trastuzumab, which improves the overall and progression-free survival of HER2+ breast cancer patients (Gianni et al., 2010; Spielmann et al., 2009; Fleming et al., 2010) and bevacizumab (Kopetz et al., 2010; Penson et al., 2010) and cetuximab (Kies et al., 2010), which are receptor-specific monoclonal antibodies that have shown very good outcomes in vascular endothelial growth factor (VEGF) receptor-positive and epidermal growth factor (EGF) receptor-positive cancer, respectively. Apart from the already discussed cancer approaches, palliative care is also very important. Its role is primarily to improve the quality of life of patients mainly through symptom control and pain management.

Considerable improvements in the classical treatment of cancer, as well as an ever increasing arsenal of prevention strategies and screening technologies, together with personalised profiling and targeting systems have allowed doctors to diagnose and treat cancer earlier in its progression and also provided novel tools for improvement of both prognostic accuracy and personalised patient treatment (Bhojwani et al., 2007).

2.2.1 Breast Cancer

Breast cancer treatment includes surgery, radiation therapy, hormonal (anti-estrogen) therapy and chemotherapy (breastcancer.org, 2012). Surgery is still the most common approach to breast cancer treatment, particularly if the tumour is localised. Sometimes chemotherapy is applied prior to surgery in order to decrease the size of the tumour. Tumour removal is done by either mastectomy or wide (local) excision, together with axillary surgery. This can be followed by breast construction if desired or needed (Cassidy et al., 2010).

Radiation therapy is used quite readily after surgery in order to eliminate cancer cells that might still be remaining in the breast tissue. It has been shown that radiation therapy can reduce the risk of breast cancer recurrence by about 70%. There are three types of radiation therapy used with external radiation being the most common, followed by internal radiation, which is not applied frequently and intraoperative radiation, which is a relatively new technique (breastcancer.org, 2012).

Chemotherapy is also used for breast cancer treatment. Usually, a combination of two or more chemotherapeutic drugs is used. Chemotherapy is applied before or after
surgery in order to reduce the size of the tumour or eliminate the remaining cells and reduce the risk of tumour recurrence, respectively. It is also applied in advanced breast cancer, where surgery and other treatments would not be effective or cannot be used. The types of bioactive agents include: anthracyclines – doxorubicin, epirubicin; alkylating agents – cyclophosphamide; anti-metabolites – 5-fluorouracil, capecitabine, methotrexate; taxanes – docetaxel, paclitaxel; vincas – vinorelbine; and platinum complexes – carboplatin, cisplatin (Cassidy et al., 2010).

Hormonal (endocrine) therapy is applied for treatment of estrogen receptor (ER)-positive breast cancers. It includes treatment with tamoxifen, progestins and aromatase inhibitors, or ovarian ablation. These treatments result in an objective response or prevent disease in about 30% of women with advanced breast cancer and in 50-60% of those with ER-positive tumours (Cassidy et al., 2010). Targeted therapy is another option for treatment. This is a very exciting and attractive type of treatment as it is directed selectively to cancer cells and limits side effects that are associated with other treatments such as chemotherapy, which harm normal healthy tissue. Some targeted therapies use antibodies, which mimic antibodies in the patients’ immune system. This type of targeted therapy is referred to as immunotherapy or immune targeted therapy (breastcancer.org, 2012). There are several targeted anti-cancer drugs such as trastuzumab, lapatinib, bevacizumab, pertuzumab and everolimus. Trastuzumab and bevacizumab have been discussed in the previous chapter. Lapatinib is a receptor tyrosine kinase inhibitor, which inhibits EGFR and HER2 and is administered to patients that are refractory to trastuzumab (Cassidy et al., 2010). Pertuzumab functions against HER2-positive breast cancers by blocking the ability of cancer cells to receive growth signals. Everolimus is an mTOR (mammalian target of rapamycin) inhibitor, which acts by depriving cancer cells of their energy (breastcancer.org, 2012). Since mTOR has been shown to regulate many cellular functions including cell growth, proliferation, motility, cell survival and apoptosis, protein synthesis, and metabolism (Laplante and Sabatini, 2012; Lebwohl et al., 2013), inhibition of mTOR with everolimus not only results in cellular energy deprivation, but ultimately leads to inhibition of global translation and impairment of tumour cell proliferation (Wullschleger et al., 2006; Lebwohl et al., 2011).
2.2.2 Prostate Cancer

There are several treatment options for prostate cancer. Similarly to breast cancer these include surgery, radiation therapy, chemotherapy, and hormone therapy, as well as active surveillance of prostate-specific antigen (PSA), which is particular for prostate cancer, or immunotherapy (Figure 11). Localised disease is treated surgically or with radiation therapy (Walsh et al., 2007) or, if the cancer is thought to be of sufficiently low risk, it may be just closely monitored (Carter et al., 2007). For up to 80% of surgically treated men, local treatment is successful in that metastatic disease does not occur within 15 years (Pound et al., 1999).

In the case of recurrence of the disease after initial therapy, prostate cancer can be treated through hormone therapy - androgen ablation, which can either be surgical castration, medical castration, androgen blockage, or maximal androgen blockage, which combines surgical or medical castration together with androgen blockade (Cassidy et al., 2010). Sometimes after initial therapy patients are only closely monitored until metastatic progression. Metastatic prostate cancer is initially treated with androgen ablation, although most patients eventually become refractory to this treatment and go on to develop the hormone-refractory metastatic disease. This stage is usually treated with chemotherapy (Petrylak et al., 2004; Tannock et al., 2004). This is, however, quite tricky especially due to the age of most patients with metastatic prostate cancer and due to the limited number of active agents. Nevertheless, mitoxantrone and docetaxel have been shown to cause a change in PSA response with symptom relief and improved median survival time (Cassidy et al., 2010).

Due to the fact that prostate cancer has limited treatment options and that associated morbidity is quite high, there has been increased interest in new treatments such as antigen-specific immunotherapy or cell-based immunotherapy, in which the patients’ immune system is targeted to induce an anti-tumour response (Drake, 2010). An example of a successful antigen-specific immunotherapy agent that has completed phase III clinical trials and is now approved for cancer treatment is sipuleucel-T, which is manufactured individually for each patient with prostate cancer (Higano et al., 2009). This agent induces T cell activation and homing to tumour lesions, which mediates an anti-tumour response.
2.2.3 Mesothelioma

Treatment of mesothelioma involves several approaches including surgery, radiation therapy and chemotherapy. Recently, several targeted therapies have been trialled. Some of these anti-cancer agents are already used for treatment of other cancer types. Surgery is performed in patients with the localised disease. These patients undergo extrapleural pneumonectomy (EPP), which is a radical excision of the entire lung, both visceral and parietal pleura, pericardium, and diaphragm with synthetic reconstruction (Cassidy et al., 2010; Mott, 2012). Lung-sparing cytoreductive surgery is a more conventional approach than EPP and is supported by some clinicians, especially when combined with chemotherapy and radiation therapy, which together is referred to as trimodality therapy (Mott, 2012). Another treatment approach is called photodynamic therapy, a light-based treatment that utilises a porphyrin-based compound, which reacts in the presence of visible light to cause direct cellular destruction and to initiate a series of apoptotic events. This therapy is only experimental for malignant pleural mesothelioma, although it has been approved for some other cancers (Friedberg, 2009).

Radiotherapy is another therapeutic approach used for reducing the chest wall masses or ameliorating pain. A more intensive type of radiation treatment with greater precision is referred to as intensity-modulated radiotherapy (IMRT). It is often used after EPP to reduce the risk of the disease recurrence. However, whether such treatment has any effective impact on survival is questionable (Mott, 2012). A third treatment
option is chemotherapy, which is often used in patients with advanced mesothelioma. Previously, chemotherapy did not show good results due to chemoresistance of mesothelioma and lack of active agents with acceptable toxicity. Nevertheless, improved results have been reported with the anti-folate pemetrexed in combination with cisplatin or carboplatin, with median survival of twelve months. There have also been some objective responses to treatment with ifosfamide and mitomycin (Cassidy et al., 2010).

Targeted therapy is also becoming an option for mesothelioma treatment. To date, however, most novel anti-cancer agents that are used in other cancer types have had a limited success in mesothelioma. Among these are bevacizumab, which is used in breast cancer and has been discussed above, vatalanib, sorafenib and sunitib, which also target the VEGF receptor and the platelet-derived growth factor (PDGF) receptor. Other targeted therapy agents include erlotinib and gefitinib, tyrosine kinase inhibitors that target EGF receptor; and ranpirnase, an amphibian ribonuclease that targets tRNA (Mott, 2012). This agent has shown an improved median survival for mesothelioma patients (Vogelzang et al., 2000).

2.3 Murine Models of Breast, Prostate Cancer and Mesothelioma

Studies at the cellular level have allowed us to establish pathways that operate in a single cell or within a population of cells. However, research in vivo (in this case using mouse models) integrates the complexity of an organ and its different cell types with the dynamic hormonal and physiological status of the whole animal. Numerous mouse models of mammary cancer that mimic various features of the human disease have been developed (Ottewell et al., 2006). Most mouse transgenic models for prevention studies have been generated through gain-of-function or knock-out of critical components of oncogenic pathways. The most commonly used mouse models for breast cancer research cover a wide range of diverse targets such as growth factors, receptors, cell cycle regulators, signal transduction pathways, cellular differentiation, oncogenes and tumour suppressor genes (Shen and Brown, 2005). Among the commonly used mouse models for breast cancer prevention studies are TGFα, ErbB-2 (wild-type and activated forms), Wnt-1, Ras, c-myc, SV40 T-antigen, p53 and cyclin D1 models (Shen and Brown, 2005).

ErbB-2 (HER2, Neu) is one of the most intensively studied genes in breast cancer biology due to the fact that overexpression and amplification of the erbB-2
oncogene has been implicated in the development of aggressive human breast cancer (Slamon et al., 1987, 1989). It is over-expressed in up to 30% of breast cancers. In the relevant animal model, the FVB/N c-neu transgenic mouse, spontaneous mammary carcinomas appear at > 6 months of age in about 70% of female mice with metastasis apparent at > 10-12 months of age (Hutchinson and Muller, 2000). This model features <1% of CSCs in the tumour formed (Dr Jane Visvader, personal communication). In contrast, the Wnt-1 mouse model contains up to 10% of CSCs within the tumour formed (Dr Jane Visvader, personal communication). The Wnt-1 gene was originally found to be activated after MMTV (mouse mammary tumour virus) infection and the resulting mice had a high incidence of mammary tumours. Use of the MMTV promoter-driven wnt-1 expression has been found to cause ductal hyperplasia in late gestation and in pre-pubertal mice (Tsukamoto et al., 1988). As a result, the wnt-1 mice develop adenocarcinomas by about 6-12 months of age (Tsukamoto et al., 1988; Kwan et al., 1992).

Several mouse models of prostate cancer exist today. However, generating these posed considerable complications as apparent differences exist between the human and murine prostate and also, importantly, the mouse prostate does not spontaneously develop neoplasia. One of the most well-known models is the TRAMP (transgenic adenocarcinoma of the mouse prostate) model (Greenberg et al., 1995; Gingrich et al., 1996), which demonstrates various stages of progressive prostate disease with focal adenocarcinomas developing between 10 and 20 weeks of age with 100% frequency (Greenberg et al., 1995). Another transgenic mouse used is the C3(1)/SV40 mouse model. This model shows a well-characterised and reliable progression of the disease from low grade PIN to high grade PIN to focal and invasive adenocarcinoma. This model gives strong support for PIN being a true precursor to carcinoma (Shibata et al., 1996, 1998). A very interesting model, which allows for studying of the neuroendocrine-derived prostate neoplasia is the CR2-T-Ag transgenic mouse model (Garabedian et al., 1998). These mice develop rapidly progressing prostate cancers, following PIN, and they metastasise despite the absence of androgen stimulation (Garabedian et al., 1998).

To date there have been several mouse models of malignant mesothelioma produced. Of these there is one transgenic mouse model, which was developed by Grippo and Sandgren (2000), which demonstrates rapid development of diffuse peritoneal malignant mesothelioma. However, unfortunately these mice are not able to
reproduce. Other mouse models of malignant mesothelioma were developed using genetically engineered animals, where heterozygous p53-deficient mice developed asbestos-induced peritoneal malignant mesothelioma (Vaslet et al., 2002), and heterozygous Nf2-deficient mice showed an accelerated rate of tumour growth and invasion of asbestos-induced malignant mesothelioma (McClatchey et al., 1998). These mesotheliomas mimicked the human disease with respect to latency, superficial growth of the tumour, shedding of tumour cells and build-up of serosanguinous ascites (Craighead and Kane, 1994). Recently, a group in the Netherlands produced the Nf2;Ink4a/Arf and Nf2;p53 conditional knock-out mice, which developed mesothelioma at high incidence and resembled human malignant mesothelioma (Jongsma et al., 2008).

Another mouse model used commonly in cancer research is the non-obese diabetic, severe combined immunodeficiency (NOD/SCID) mouse or the nude (BALB c nu/nu) mouse. Purified cancer cells, CSCs or spheres (clusters of cells grown in culture, which maintain stem cell characteristics, such as mammospheres, prostate spheres or mesospheres) derived from human breast and prostate cancer and mesothelioma cell lines can be implanted into these animals, usually subcutaneously, to form tumours (xenografts).

The use of these models makes it possible to perform pre-clinical chemotherapeutic, chemopreventative and genetic studies in vivo, as well as to test gene delivery systems and the identification of tumour and metastatic suppressor and inducer genes. Importantly, relevant models can help validate results obtained in cell culture studies on the in vivo level, following which translation to the pre-clinical/clinical stage may be initiated.

2.4 Cancer Stem Cells
In the second half of the 19th century, it was first proposed that cancer might arise from a rare cell sub-population with stem cell-like properties. However, it was not until recently that progress in stem cell biology gave new impetus to the ‘cancer stem cell hypothesis’ (Wicha et al., 2006). Stem cells are defined as cells that have the ability to undergo self-renewal and to generate mature cells through differentiation (Reya et al., 2001; Liu et al., 2005). Self-renewal can be either symmetric or asymmetric (Figure 12). Asymmetric division is cell division in which a stem cell produces an exact copy of itself as well as a daughter cell, which then differentiates into a mature cell. For example, this process can occur in tissue homeostasis. Symmetric division is cell
division where a stem cell gives rise to two identical copies of itself and these cells do
not differentiate but remain as stem cells. This process can be observed during stem cell
expansion, in early stages of organogenesis before the cells become committed and
terminally differentiated and it is also very important during carcinogenesis (Liu et al.,
2005; Wicha et al., 2006).

Self-renewal is a crucial stem cell function, which is tightly regulated in normal
organogenesis. Dysregulation of this process may be one of the crucial events, which
leads to carcinogenesis. In fact, cellular signalling pathways and transcription factors
that are responsible for self-renewal of normal stem cells and also for regulating the
balance between self-renewal and differentiation have all been implicated in
carcinogenesis (Reya et al., 2001). These pathways include the Hedgehog, Notch and
Wnt signalling routes. The transcription factor B lymphoma Mo-MLV insertion region
1 (Bmi-1) has also been associated with the process (Liu et al., 2005).
Figure 12: Stem cells in normal development, tissue homeostasis, and carcinogenesis. A) During normal development, symmetric stem cell self-renewal results in cell expansion. This process is highly regulated by components of the stem cell niche. Stem cells differentiate into a transiently amplifying population that goes through further proliferation and lineage commitment followed by cell migration, terminal cell differentiation, and apoptosis of fully differentiated cells. B) During normal tissue homeostasis, asymmetric self-renewal of stem cells results in stem cell maintenance. Proliferation and differentiation of transient amplifying progenitor cells replaces normal cell loss, which results in tissue homeostasis. C) Carcinogenesis may be initiated by stem cell expansion via symmetric self-renewal. Unlike normal organogenesis, this process is dysregulated, which results in cancer stem cell expansion. Aberrant differentiation of these cells creates tumour heterogeneity. It is possible that further mutations or epigenetic changes accompany tumour invasion and metastasis. Metastases require the dissemination of cancer stem cells that may remain dormant and later on be reactivated resulting in tumour recurrence. In contrast, dissemination of differentiated tumour cells produces only cells that do not progress (Wicha et al., 2006).
The cancer stem cell (CSC) theory is based on the concept that a defined subset of cancer cells has the ability to promote the growth of a tumour and to metastasise. These CSCs can give rise to cancer cell progeny that are more differentiated and eventually destined to stop proliferating or die with limited ability or no ability to undergo further cell division (Lobo et al., 2007). This theory is centred on the notion that some elements of the cellular hierarchy in normal tissues are maintained in tumours and it is also supported by the fact that stem cells, due to their long-lived nature, are prone to accumulate various mutations, which are needed for carcinogenesis (Mackenzie, 2006; Paguirigan et al., 2006). A prime example of tumour formation caused by acquiring mutations in stem cells comes from Hiroshima and Nagasaki. Here, many women exposed to atomic bomb radiation developed breast cancer approximately 20 to 30 years after the exposure, and the mutations found in these women’s breast cancers were consistent with those that are known to be induced by radiation (Little and Boice, 1999). Moreover, studies of women exposed to radiation during late adolescence revealed the highest susceptibility to breast cancer development and, not surprisingly, this is thought to be the period when the mammary gland contains the highest number of stem cells (Smith and Chepko, 2001).

Other evidence indicates that stem cells may play an important role in carcinogenesis due to the observation that CSCs share many crucial characteristics with normal stem cells. These include the ability to self-renew and differentiate, high levels of telomerase, higher levels of DNA repair, activation of anti-apoptotic pathways, increased membrane transporter activity including the ATP-binding cassette (ABC) drug transporters, providing for an increased level of drug resistance, the ability to migrate and metastasise, and hence form secondary tumours and, finally, the ability to evade the immune system (D’Amour and Gage, 2002; Singh et al., 2004; Wicha et al., 2006; Lou and Dean, 2007; Tang et al., 2007; Hanahan and Weinberg, 2011; Scheel and Weinberg, 2012). In addition to CSCs, there are also cells or cellular structures that support tumour development (carcinogenesis) by promoting angiogenesis (Rak, 2006). This process results in the increased delivery of oxygen and nutrition to the tumour, so that it can grow and expand.

An important aspect worth mentioning here is the concept of the so-called ‘cell-of-origin’ and CSCs. A cell of origin is the cell, which acquires the first cancer-promoting mutation or mutations, although it does not have to be a CSC. This means that the cell-of-origin model refers to cancer-initiating cells and the CSC model refers to
cancer-propagating cells (Visvader, 2011). Nevertheless, there is increasing evidence, mainly from lineage-tracing studies, that the cell-of-origin is either a stem cell or a progenitor cell. The cell-of-origin in prostate cancer has been identified as a stem cell (Wang et al., 2009), in intestinal cancers these cells comprise two types of distinct crypt stem cells (Barker et al., 2009). For glioblastomas, stem cells or multipotential neural progenitors in the subventricular zone have been implicated, although this is very complex and further research is needed (Holland et al., 2000; Merkle et al., 2007). In basal-like breast cancers the luminal progenitor cell has been implicated as the cell-of-origin (Lim et al., 2009; Molyneux et al., 2010). Despite the fact that the cancer-initiating cell might not always be a stem cell, CSCs are crucial and essential for the propagation and maintenance of a tumour.

The epithelial-mesenchymal transition (EMT) is an important process in which epithelial cells acquire mesenchymal properties that allow them to invade the surrounding tissue and subsequently disseminate to distant sites (this is often termed colonisation), where they form micro- and macroscopic metastases (Scheel and Weinberg, 2012). In order to successfully form new colonies (micro- and macrometastases), the disseminated tumour cells must acquire the ability to survive and proliferate, traits which are associated with CSCs. Interestingly, molecular links between the EMT programme and stem cell fate (for example self-renewal) are starting to emerge, indicating a connection between passage through the EMT and acquisition of stem cell traits, and therefore suggesting that EMT programmes may play a critical role in metastasis. In the setting of tumour development, the EMT-inducing signals seem to originate in the adjacent stroma. These extracellular signals that are able to induce EMTs in various epithelial cell types, include TGF-β ligands, Wnt, Notch, Sonic Hedgehog and growth factors that activate tyrosine kinase receptors, such as EGF (Oft et al., 1996; Thiery et al., 2009). Quite importantly, hypoxia has also been implicated in the induction of EMT, with hypoxia-inducible factor-1α (HIF1-α), directly activating Twist1 in head and neck squamous carcinomas (Yang et al., 2008). Once the tumour cells have translocated to distant sites, the process is reversed and these cells undergo the mesenchymal-epithelial transition (MET), which allows them to regain their epithelial phenotype. It is believed that this reversion is quite critical for successful colonisation (Chaffer et al., 2006; Spaderna et al., 2006). Scheel and Weinberg discuss the notion that activated EMT programs in tumour cells lead these cells into the stem-cell state, giving them the ability to migrate and metastasize and hence form new
tumours. It is, however, also plausible that it is in fact CSCs themselves that are more likely to undergo EMT, that metastasis-forming ability is limited to CSCs (Brabletz et al., 2005). So, it is a question whether EMT programs promote stem cell properties of tumour cells or whether EMT is a feature of CSCs. More studies need to be done in this area. Nevertheless, EMT is an important factor in neoplastic diseases and anti-cancer therapies have to take this into consideration.

CSCs have now been identified in different cancers including leukaemias (Lapidot et al., 1994; Bhatia et al., 1998), multiple myelomas (Park et al., 1971), neoplasias of the nervous system (Singh et al., 2003; Hirschmann-Jax et al., 2004; Singh et al., 2004; Piccirillo et al., 2006), colorectal, pancreatic, prostate and hepatocellular carcinomas (Collins et al., 2005; O’Brien et al., 2007; Suetsugu et al., 2006; Ricci-Vitiani et al., 2007; Yin et al., 2007), breast and lung cancers (Al-Hajj et al., 2003), melanomas (Fang et al., 2005), osteosarcomas (Wang et al., 2009), mesotheliomas (Neuzil et al., unpublished) and other tissues prone to cancer development. Within these tumours CSCs sometimes form a small sub-population referred to as the side-population due to the fact that these cells can actively exclude fluorescent DNA staining dyes (Guo et al., 2004). They are able to do so because of the increased expression of the ABC drug transporters.

A variety of markers have been used to characterise CSCs. Several markers can be used to define CSCs derived from different tissues, other markers are tissue-specific (Table 5). In particular, these include CD133 and CD44 surface proteins and several intrinsic markers (e.g. Oct-4 or ALDH1). Overexpression of CD44 has been found in the CSC population in breast (Al-Hajj et al., 2003), prostate (Collins et al., 2005) and pancreatic cancer (Li et al., 2007). The CD44 protein is involved in cell-cell and cell-matrix interactions, and CD44+ breast cancer cells exert more pronounced TGF-β signalling (Shipitsin et al., 2007).
In normal breast tissue, there is a side-population that has been phenotypically
characterised as Lin⁻/CD29⁺/CD24⁻/low (Shackleton et al., 2006; Stingl et al., 2006). A
single cell from this side-population was able to reconstitute a complete mammary
gland in vivo when implanted into the mammary tissue of the MMTV-wnt-1 mice. Due
to the fact that breast CSCs feature characteristics of normal mammary stem cells,
including expression of specific markers, this process would appear to have important
implications for tumourigenesis. Another type of side-population has been discovered in
haematopoietic cells and it has the Lin⁻/CD34⁺/CD38⁻ genotype. These cells were
capable of re-populating the NOD/SCID mice (Bhatia et al., 1998).

Recently it has been found that diverse CSCs, especially of epithelial origin, in
most cases share a unifying phenotype with high levels of CD133 expression
(Richardson et al., 2004; Mehra et al., 2006). CD133, also known as prominin-1
(Miraglia et al., 1997; Weigmann et al., 1997; Corbeil et al., 2001), is a cell-surface

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Table 5: Cell surface markers associated with human CSCs.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>CSC markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>Stro1⁺, CD105⁺, CD44⁺</td>
<td>(Gibbs et al., 2005)</td>
</tr>
<tr>
<td>Brain</td>
<td>Musashi-1⁺, nestin⁺, CD133⁺</td>
<td>(Singh et al., 2004)</td>
</tr>
<tr>
<td>Breast</td>
<td>CD44⁺, CD29⁺, CD24⁺/low</td>
<td>(Al-Hajj et al., 2003; Ginestier et al., 2007; Wright et al., 2008)</td>
</tr>
<tr>
<td>Colon</td>
<td>CD133⁺</td>
<td>(O’Brien et al., 2007; Ricci-Vitiani et al., 2007)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>EpCAM(high), CD44⁺, CD166⁺</td>
<td>(Dalerba et al., 2007)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>CD44⁺</td>
<td>(Prince et al., 2007)</td>
</tr>
<tr>
<td>Hepatocellular</td>
<td>CD133⁺</td>
<td>(Suetsugu et al., 2006)</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>CD34⁺, CD38⁻, CD44⁺</td>
<td>(Bonnet and Dick, 1997)</td>
</tr>
<tr>
<td>Lung</td>
<td>Sca1⁺, CD45⁻, Pecam⁻, CD34⁺</td>
<td>(Kim et al., 2005)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CD20⁺, CD133⁺, ABCG2⁺</td>
<td>(Fang et al., 2005; Monzani et al., 2007)</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>CD24⁺, Oct-4⁺, ABCG2⁺</td>
<td>(Neuizil et al., unpublished)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>CD133⁺, ABCG2⁺, CD24⁺, CD44⁺, ESA⁺</td>
<td>(Li et al., 2007; Olempska et al., 2007)</td>
</tr>
<tr>
<td>Prostate</td>
<td>CD44⁺, α₂β₁(high), CD133⁺</td>
<td>(Collins et al., 2005; Patrawala et al., 2006; Miki et al., 2007)</td>
</tr>
</tbody>
</table>
glycoprotein consisting of five trans-membrane domains and two large glycosylated extracellular loops. CD133 was first isolated from haematopoietic stem cells and was found to localise to membrane protrusions (Miraglia et al., 1997; Corbeil et al., 2001). Its function has not yet been established, although, it could participate in the regulation of membrane topology (Röper et al., 2000; Shmelkov et al., 2005).}

**Figure 13: A structure model of CD133.** This protein comprises an extracellular N-terminus, 5 hydrophobic trans-membrane domains, 2 small cytoplasmic loops, 2 large extracellular loops and a cytoplasmic C-terminus (adapted and modified from Weigmann et al., 1997; Miraglia et al., 1997; Röper et al., 2000; Corbeil et al., 2001; Fargeas et al., 2003; Fargeas et al., 2006).

Several studies have shown that CD133\(^+\) cancer cells can give rise to tumours *in vivo*. One study demonstrated that subcutaneous implantation of purified CD133\(^+\) Huh-7 cells in NOD/SCID mice, resulted in the formation of tumours, while CD133\(^-\) Huh-7 cells did not form tumours (Suetsugu et al., 2006). Another study showed that whereas as many as 10\(^7\) cultured hepatocarcinoma cells were required to form tumours in NOD/SCID mice, only 10\(^3\) or less CD133\(^+\) cells were required to form tumours (Yin et al., 2007). Similar results were obtained using breast CSCs (Wright et al., 2008). Recently the existence of CD133\(^+\) cells in human brain cancers has been demonstrated. These cells possess differentiation and self-renewal capacities, and can initiate tumour growth *in vivo*, whereas the corresponding CD133\(^-\) cells cannot (Singh et al., 2004). *In vivo* injection of as few as one hundred CD133\(^+\) cells was able to regenerate a serially transplantable phenocopy of the original tumour in the brains of NOD/SCID mice.
Quite recently Shmelkov and colleagues demonstrated that CD133− cells can also form tumours in NOD/SCID mice. However, in this study they also showed that CD133+ cancer cells have much greater metastatic potential than their CD133− counterparts (Shmelkov et al., 2008). For these reasons, high level of CD133 expression has been accepted as an important CSC marker for a number of different cell lineages (Shmelkov et al., 2005), and expression of CD133 in peripheral blood correlates with bone metastasis in cancer patients (Mehra et al., 2006). Its role as a marker has been questioned recently and other markers are also used (e.g. CD44, Oct-4, ALDH1), although for some types of cancer, such as glioblastomas, CD133 is still the best marker to use.

2.5 Relevant Signalling Pathways

Several signalling pathways play a key role in the development and regulation of stem cell self-renewal and differentiation. They include the Hedgehog, Notch and Wnt pathways as well as the transcription factor Bmi-1 with a similar role (Liu et al., 2005). These pathways are not only involved in stem cell self-renewal but also in carcinogenesis, providing support for the notion that carcinogenesis may result from deregulation of self-renewal pathways of normal (somatic) stem cells. With this in mind, cancer can be considered as a disease of unregulated self-renewal (Reya et al., 2001).

The Hedgehog (HH) pathway (Figure 14) was first identified in Drosophila, where it is required for early embryo patterning. It regulates transcription of several genes, including those controlling cell proliferation such as cyclin D, cyclin E, and Myc, components of the epidermal growth factor pathway as well as angiogenesis modulators such as the PDGF and the VEGF (Liu et al., 2005; Evangelista et al., 2006). Recent studies have indicated that HH signalling is also very important in embryonic mammary gland induction, ductal morphogenesis and alveolar development. A critical role for HH signalling in mediating epithelial stromal interactions during ductal development has been shown by the genetic analysis of two HH signal transduction network genes, Ptch1 and Gli-2. Disruption of either gene leads to similar, yet distinct defects in ductal morphogenesis that are mainly ductal dysplasias, similar to the hyperplasias of the human breast (Liu et al., 2005). The importance of HH signalling in carcinogenesis has been demonstrated by the fact that many of the target genes are oncogenes, including Smo, Shh, Gli-1 and Gli-2, while Ptch1 is a tumour suppressor. Mutations in these
genes have been commonly linked to the development of many cancers shown to depend on activated HH signalling (Lewis and Veltmaat, 2004).

Mutations in the HH pathway were first described in the Gorlin syndrome and basal skin carcinoma. More recently, an important role for HH signalling has been shown in medulloblastoma, prostate, and pancreatic carcinomas (Karhadkar et al., 2004; Olsen et al., 2004). Similarities between HH mutation-induced ductal dysplasias and human breast pathologies suggest a general role for altered HH signalling in the development of mammary cancer. There is also evidence that altered HH signalling has a direct role in the neoplastic progression of the mammary gland. One study showed Ptc1 mutations in two out of seven human breast cancers (Xie et al., 1997).
Figure 14: A schematic diagram of the HH signalling pathway. The Sonic HH (Shh), Indian HH (Ihh) and Desert HH (Dhh) ligands are secreted by signalling cells. They bind to the trans-membrane receptor patched (Ptch) in the responding cells. In the absence of ligands, Ptch binds to Smoothened (Smo), blocking its function. However, in the presence of ligands, this inhibition is relieved and Smo initiates a signalling cascade that results in the release of transcription factors of the Gli family from the cytoplasmic proteins fused (Fu) and suppressor of fused (SuFu). In the inactive situation, SuFu prevents Gli from translocating to the nucleus. Following activation, Fu inhibits SuFu and Gli is released. The Gli proteins translocate into the nucleus where they regulate transcription of target genes. The red lines and the compounds in red show the inhibitors of this pathway (Liu et al., 2005).

The Notch signalling pathway (Figure 15) plays an important role in the regulation of cell fate in a variety of tissues. There are four known homologues in mammals, Notch1 to Notch4, which are expressed in a variety of stem or early progenitor cells (Mumm and Kopan, 2000). Activation of the Notch pathway causes changes in the cell fate, mainly regulating self-renewal of stem cells or differentiation
along a given lineage (Krause, 2002). It has been established that the Notch pathway is involved in the normal development of the mammary gland (Liu et al., 2005). One study showed that in vitro, overexpression of the constitutively active form of Notch4 inhibited the differentiation of normal breast epithelial cells. Weijzen et al. (2002) showed that Notch1 is a downstream effector of oncogenic Ras and that its signalling activation maintains the neoplastic phenotype in human Ras-transformed cells. Using a mammosphere system it was found that Notch signalling is active in a number of distinct developmental phases of the mammary gland and that Notch has a role in the regulation of asymmetric cell fate decisions (Liu et al., 2005). These findings further confirm the role of Notch in promoting self-renewal of mammary stem cells, as well as functioning as a proto-oncogene (Uyttendaele et al., 1998; Soriano et al., 2000). Therefore, it can be suggested that abnormal Notch signalling may be involved in carcinogenesis via the deregulation of normal mammary stem cell self-renewal.

Deregulation of Notch activity has also been shown for other cancer types such as head and neck, renal, pancreatic, ovarian and prostate cancers (Santagata et al., 2004; Villaronga et al., 2008), as well as leukaemias and malignant mesothelioma (Miele, 2006; Graziani et al., 2008). Wang et al. (2010) demonstrated that downregulation of Notch1 or Jagged1 resulted in the inhibition of cell growth, migration and invasion, and induced apoptosis, which was in part due to inactivation of the Akt, mTOR, and NF-κB signalling pathways, suggesting that inactivation of Notch signalling pathways could also be a potential target for the treatment of prostate cancer.
Figure 15: A schematic diagram of the Notch signalling pathway. When the ligand DSL binds to the protein Fringe, Notch signalling is triggered by activating Notch receptors through serial cleavage events involving members of the ADAM ('A Disintegrin And Metalloproteinase') protease family, as well as an intra-membrane cleavage regulated by γ-secretase (presenilin). This intra-membrane cleavage is followed by translocation of the intracellular domain of Notch to the nucleus, where it acts on downstream targets. CBF - C promoter binding factor, HDAC - histone deacetylase, HAT - histone acetyltransferase (Liu et al., 2005).

The Wnt signalling pathway (Figure 16) plays an important role in cell fate determination in several tissues, including the mammary gland. There are two Wnt signalling pathways, the canonical and the non-canonical one. The Wnt canonical pathway functions in cell fate determination and the Wnt non-canonical pathway in the control of cell movement and tissue polarity (Katoh and Katoh, 2007). Recently, it has been shown that the Wnt pathway is crucial for different aspects of vertebrate embryo development (Veeman et al., 2003). Also, a number of studies have established a direct role of Wnt signalling in the self-renewal of haematopoietic, gut and epidermal stem cells (Brittan and Wright, 2002; Reya et al., 2003). Experiments in transgenic mice
revealed that activation of the Wnt pathway in epidermal stem cells leads to the development of epithelial cancers (Honeycutt and Roop, 2004), indicating a direct role for dysfunction of this pathway in carcinogenesis. Likewise, dysregulation of the WNT/β-catenin pathway is believed to contribute to prostate cancer progression, by β-catenin associating with the androgen receptor (Cronauer et al., 2005; Kypta and Waxman, 2012). This pathway also affects proliferation, differentiation and epithelial-mesenchymal transition (EMT) of prostate cells, and this is believed to regulate the invasive behaviour of these tumour cells.

The WNT/β-catenin pathway can be controlled by secreted Wnt antagonists, of which many are downregulated in cancer (Kypta and Waxman, 2012). In a different study, Abutaily et al. (2003) demonstrated that β-catenin accumulates in the nucleus in malignant mesotheliomas and that the adenomatous polyposis coli gene (APC) expression was altered in some mesotheliomas, suggesting that a truncated APC gene product may contribute to the abnormal Wnt signalling and dysregulation of cell proliferation in this type of cancer. Furthermore, it has been demonstrated that there is upregulation of the uncomplexed transcriptionally active form of β-catenin without mutations affecting the downstream components in breast cancer (Bafico et al., 2004). Another study showed that overexpression of Wnt ligands in mammary stroma or activated β-catenin in the mammary epithelium leads to increased numbers of mammary stem cells (Liu et al., 2004), further supporting a role for Wnt signalling in stem cell self-renewal. Studies linking this process to mammary carcinogenesis include those showing that mammary stem cells and progenitors may be targets for oncogenesis via components of the Wnt1 signalling pathway (Li et al., 2003). Hence, the Wnt pathway has an important role in mammary carcinogenesis, as well as the progression of prostate cancer and mesothelioma.
Figure 16: A schematic diagram of the Wnt signalling pathway. A. The canonical Wnt/β-catenin pathway. In the canonical pathway, Wnt signalling binds Frizzled (Fz) and the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) co-receptors to activate Dishevelled (Dsh). Once activated, Dsh inhibits the activity of the β-catenin destruction complex (adenomatous polyposis coli, APC), axin, and glycogen synthase kinase-3 (GSK-3), which phosphorylates β-catenin in the absence of the ligands. Following β-catenin stabilisation, the protein moves into the nucleus, where it recruits trans-activators to the high mobility group (HMG)-box DNA-binding proteins of the lymphoid enhancer factor/T cell factor (LEF/TCF) family. B. The non-canonical Wnt signalling pathway. Frizzled receptors and the proteoglycan co-receptor Knypek are needed to activate Dsh. Once Dsh is activated, it activates Rho with the aid of Daam1. Dsh can also stimulate calcium flux and activation of the calcium-sensitive kinases protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII). Activation of this pathway results in the induction of a variety of complex and dynamic cellular responses (Liu et al., 2005).

Bmi-1, an important transcription repressor, should be also mentioned in this context. It belongs to the polycomb group (PCG) of transcription factors and was first identified in B-cell lymphoma (Alkema et al., 1993). It has been established that Bmi-1 is a key regulator of self-renewal of both normal and leukaemic stem cells (Lessard and
Sauvageau, 2003; Park et al., 2003). In addition, it was shown that Bmi-1 is very important in neuronal stem cell self-renewal (Molofsky et al., 2003). Due to the fact that Bmi-1 was shown to be overexpressed in a number of human breast cancer cell lines, a link between Bmi-1 and mammary carcinogenesis was suggested. Furthermore, one study showed that Bmi-1 regulates telomerase expression in mammary epithelial cells, providing further evidence indicating that Bmi-1 plays a role in mammary carcinogenesis (Dimri et al., 2002). Correspondingly, a different study revealed that the Bmi-1-driven pathway was engaged in both normal stem cells, as well as a highly malignant subset of human cancers including prostate, breast, lung, ovarian and bladder cancers, and also lymphoma, mesothelioma, medulloblastoma, glioma, and acute myeloid leukaemia, uniformly exhibiting a marked propensity toward metastatic dissemination as well as a high probability of unfavourable therapeutic outcome (Glinsky et al., 2005).

From the examples above, it is quite clear that the three signalling pathways and the Bmi-1 transcription factor have a key role in cell fate, especially in regulating self-renewal and development, and that their dysregulation will have a great impact on carcinogenesis. However, these pathways also interact with each other and hence may be complicit in development of neoplasias that are more aggressive and, from the clinical point of view, more difficult to treat. For example, activation of two markers of active Wnt signalling (β-catenin and LEF-1) in the skin is associated with Notch-dependent transformation (Kopper and Hajdu, 2004). In addition, it is possible that activation of Smo may instigate processes during which transcription factors, which belong to the Gli family, are activated, and these, in turn, modulate transcription of the Ptc and Wnt pathway (Kopper and Hajdu, 2004). Wnt signalling has also been observed in human basal carcinomas, indicating that the process of tumour formation is mediated through interactions of different signalling pathways that regulate organ development during embryogenesis (Liu et al., 2005). Finally, it was demonstrated that overexpression of Bmi-1 correlated with expression of Ptc and SuFu, suggesting activation of the HH pathway in Bmi-1 overexpressing tumours (Leung et al., 2004).

2.6 Apoptotic Pathways
Apoptosis is a regulated cell death that acts as a major control mechanism by which cells die if DNA damage is not repaired (Lowe and Lin, 2000). The morphological changes that define apoptosis include cell shrinkage, membrane blebbing, apoptotic
body formation, chromatin condensation and nuclear fragmentation. There are two major apoptotic pathways, the extrinsic (or receptor-mediated) and intrinsic (or mitochondrial) pathway (Figure 17). The extrinsic pathway is death receptor-dependent, however, it can also be ‘dependence’-receptor dependent. In this case the pro-apoptotic signal is transmitted by the so called ‘dependence receptors’, including netrin receptors such as UNC5A-D and deleted in colorectal carcinoma (DCC) (Mehlen and Bredesen, 2011). Following ligand deprivation-induced dependence receptor signalling either directly or through mitochondrial outer membrane permeabilisation (MOMP), the caspase-9 and caspase-3 cascade is activated, leading to cell death (Galluzzi et al., 2012). The death receptor-dependent pathway is induced upon ligand binding to cognate death receptors or upon cell-to-cell contacts (Zapata et al., 2001). After the initiation step, and following DISC (death-inducing signalling complex) formation, the initiator caspases (for the extrinsic pathway caspase-8 and -10) are activated, with ensuing activation of effector caspases (caspase-3, -6 and -7 for both pathways). These then cleave the so called ‘death substrate’, which includes regulatory and structural molecules including protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonucleases (the CIDE family), which results in the final destruction of ‘house-keeping’ cellular functions and culminates in cell death (Thornberry and Lazebnik, 1998; Mancini et al., 1998). In some cells such as hepatocytes and pancreatic β cells (termed ‘type II cells’) (Scaffidi et al., 1998; Barnhart et al., 2003), caspase-8 activation leads to the cleavage of Bid, which is followed by MOMP and activation of caspase-9 with ensuing activation of caspase-3 (Li et al., 1998; Luo et al., 1998; Galluzzi et al., 2012).

The intrinsic pathway is mitochondria-dependent. It is often triggered as a result of DNA damage, starvation, activation of oncogenes, UV radiation or other internal stimuli such as generation of reactive oxygen species (ROS) that affect mitochondria. Mitochondrial insult usually results in the release of cytochrome c into the cytosol, where it interacts with Apaf-1 and pro-caspase-9 (initiator caspase for the intrinsic pathway) to form the apoptosome and activate caspase-9. Active caspase-9 then cleaves and activates caspase-3, which subsequently degrades regulatory and structural molecules leading to induce the final demise of the cell (Reed, 1997). Activation of caspase-3 is the point where both apoptotic pathways converge to execute a final common death pathway (Ghobrial et al., 2005). The intrinsic apoptotic pathway can also function in a caspase-independent manner. In this scenario the apoptosis-inducing factor
(AIF) and endonuclease G (ENDOG) relocate to the nucleus and mediate large-scale DNA fragmentation (Joza et al., 2001; Li et al., 2001). The serine protease high temperature requirement protein 2 (HTRA2) has also been shown to take part in the caspase-independent intrinsic apoptosis. It acts by cleaving a wide array of cellular substrates, including cytoskeletal proteins (Hegde et al., 2002; Vande Walle et al., 2007; Galluzzi et al., 2012).

Figure 17: Two major apoptotic pathways: extrinsic and intrinsic. The extrinsic pathway, as shown, is triggered through the Fas death receptor. The intrinsic pathway is triggered as a result of DNA damage, starvation, activation of oncogenes, UV radiation and other internal stimuli that affect mitochondria and subsequently promote cytochrome c release. Both pathways converge to trigger the final common pathway involving the activation of caspases that cleave regulatory and structural molecules, which lead to physical death of the cell (Ghobrial et al., 2005).

ROS are formed as a natural by-product of the normal oxygen metabolism. Increase in the level of ROS creates oxidative stress, which causes cell damage with ensuing cell death by activating various signalling pathways that in most cases converge at apoptosis induction (Chandra et al., 2000; Mascareno et al., 2001). While the signalling mechanisms have not been completely unravelled, mitochondria play a key role in the oxidative stress-induced apoptotic pathways (Mignotte and Vayssiere, 1998).
Mitochondria, organelles vital for cellular energy homeostasis, produce ROS in the mitochondrial respiratory chain (Chakraborti et al., 1999). Recent studies showed that ROS generation greatly depends also on complex II of the respiratory chain (McLennan and Degli Esposti, 2000; Adam-Vizi and Chinopoulos, 2006), making it very important for apoptosis and cell death (Albayrak et al., 2003; Miyadera et al., 2003; Dong et al., 2008; Dong et al., 2009).

A key extrinsic apoptotic pathway is the TNF-related apoptosis-inducing ligand (TRAIL)-mediated process (Figure 18). TRAIL is one of the major denominators of anti-tumour immunity. Present on the surface of immune cells as a membrane protein or secreted in a soluble form, it induces apoptosis by binding to the death receptors TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) or TRAIL receptor 2/death receptor 5 (TRAIL-R2/DR5) (Tomasetti et al., 2006). Binding of TRAIL to the receptors induces trimerisation, which is followed by recruitment of down-stream mediators of apoptosis via the help of adaptor proteins such as FADD (Fas-associated death domain), resulting in the formation of DISC (Curtin and Cotter, 2003). Following the recruitment of pro-caspases-8 and -10 by FADD to the DISC, activation of caspase-8 and -10 occurs via auto-proteolytic cleavage. Caspase-8 and -10 then directly cleave and activate effector caspases, leading to cell death via the final common pathway. In some cases, active caspase-8 activates a small protein called Bid, which in turn mediates Bax and/or Bak mitochondrial pore formation. Once these pores are formed, cytochrome c can translocate into the cytoplasm and take part in the apoptosome formation. In this way extrinsic and intrinsic apoptotic pathways are interconnected. Their tightly regulated interplay can result in a more efficient apoptotic response.

TRAIL-mediated apoptosis shows a high level of specificity for cancer cells. Normal cells often express the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), to which TRAIL can bind. Binding to the DcRs will not recruit FADD, so that the downstream cell signalling events leading to apoptosis will not be triggered (Merino et al., 2006). Cancer cells usually do not express the DcRs and hence are more susceptible to TRAIL-mediated apoptosis, unless a different resistance mechanism develops. However, recent studies have shown that cancer cell resistance to TRAIL-induced apoptosis, at least in some cases, may be due to increased levels of expression of the DcRs (Merino et al., 2006). Recently, it has been indicated that cancer cell resistance to TRAIL-mediated cell death may be due to upregulation of the anti-apoptotic FLICE-
like inhibitory protein (FLIP) (Irmler et al., 1997; Wicha et al., 2006; Gesericke et al., 2008; Zobalova et al., 2008).

Figure 18: TRAIL-mediated apoptotic pathway (Falschlehner et al., 2007).

2.7 Survival Pathways

p53, the product of the tumour suppressor gene TP53, is a protein of great importance for carcinogenesis. It has been extensively studied both on the structural and functional level, mainly because of its vital role as a tumour suppressor in mammals (Royds and Iacopetta, 2006). Loss or mutation of p53 is strongly associated with increased susceptibility to cancer, since p53 protects from the conversion of a cell to the malignant phenotype (Vogelstein et al., 2000). p53 can be activated by diverse stimuli and has a variety of functions (Figure 19). One of the most important roles for p53 involves both the positive and negative transcriptional regulation of the expression of a large and highly diverse group of responsive genes (Laptenko and Prives, 2006). Many of them play a crucial role in mediating cell-cycle arrest, senescence and apoptosis (thus far the best understood activities of p53). However, it is now apparent that the ability of p53 to influence gene expression has wide-ranging effects (Vousden and Lane, 2007). Results from many studies have documented that p53 has a role in the regulation of cell
survival and oxidative stress (Bensaad and Vousden, 2005), cellular senescence (Kortleverm et al., 2006), angiogenesis (Toeodoro et al., 2006), differentiation (Murray-Zmijewski et al., 2006), regulation of glycolysis (Bensaad et al., 2006; Matoba et al., 2006) and autophagy (Crighton et al., 2006), invasion and motility (Roger et al., 2006), the repair of genotoxic damage (Gatz and Wiesmuller, 2006), as well as bone remodelling (Wang et al., 2006).

Hence, activation of p53 can result in various cellular responses, and it is probable that these responses are induced by different stress signals. It has been suggested that p53 can play a part in determining which response is induced by differentially activated expression of target-genes (Vousden and Lane, 2007). The role of p53 in tumour suppression, development and ageing is therefore likely to depend on which cellular response is activated and on the context in which the activation occurs.

**Figure 19: Activation and functions of p53.** p53 has a key role in integrating the cellular responses (purple boxes) to diverse types of stress (blue boxes) (Vousden and Lane, 2007).

p53 is activated by both constitutive stress (encountered during normal growth and development) and by acute stress signals (such as those that would be associated with oncogenic progression and other types of highly stressful signals) (Vousden and Lane, 2007). As can be seen in Figure 20, p53 plays a significant role in decreasing oxidative damage and repairing low levels of DNA perturbation. It is these activities of p53 that have the main role in survival and health of each normal cell. p53 also participates in preventing the acquisition of tumorigenic mutations, and in this regard most likely contributes to the overall longevity and normal development. On the other
hand, activation of p53 by high or acute stress results in activation of apoptotic cell death, leading to elimination of the damaged cell (Vousden and Lane, 2007).

Induction of apoptosis is one of the most dramatic responses to p53 activation. It is now well established that p53 functions in both the extrinsic and intrinsic apoptotic pathways when these are induced by stress signals (Figure 21A) (Green, 1998). In response to acute stress, p53 induces the expression of genes that encode apoptotic proteins with a function in both pathways. However, it appears that p53 predominantly influences proteins of the intrinsic pathway, including target genes that encode the BH3-only proteins NOXA and PUMA (Jeffers et al., 2003; Villunger et al., 2003). Recent research has also suggested direct binding of p53 to Bcl-2 family proteins such as Bcl-X_L (Figure 21B) (Laptenko and Prives, 2006; Vousden and Prives, 2009). This function of p53 results in removal of cells that have acquired oncogenic alterations. However, it also mediates accompanying detrimental effects of stress-induced toxicity, such as radiation sickness, neurodegenerative disease and premature ageing.

Figure 20: p53-mediated regulation of life and death in response to different levels of stress signals (Vousden and Lane, 2007).
Figure 21: A model of p53-mediated apoptosis. A, classical understanding of the role of p53 in both the extrinsic and intrinsic apoptotic pathways. The two pathways converge by means of the Bid protein. p53 target genes are shown in red (Haupt et al., 2003); B, direct interaction of p53 with Bcl-2 family proteins including Bcl-XL (adapted from Amaral et al., 2010).
It is therefore obvious that the p53-activating signals function through different pathways and that the response to p53 can vary depending on cell type, environment, and other contributing factors (Vousden and Lu, 2002).

2.8 Cancer Stem Cells and the Immune System
Carcinogenesis is the result of accumulation of mutational and epigenetic changes, which change normal cell growth control, survival and pro-apoptotic pathways. These newly formed malignant cells are morphologically distinct and have newly acquired features including the ability to evade apoptosis, proliferate out of control, invade the surrounding tissue, induce angiogenesis and eventually metastasise (Hanahan and Weinberg, 2011). During the early stages of carcinogenesis, if cellular proliferation and division become abnormal, malignant transformation can be attenuated and the transformed cells eliminated using a variety of intrinsic tumour suppressor mechanisms such as apoptosis, repair or senescence (Smyth et al., 2006).

Not all malignant cells get destroyed during the early stages of carcinogenesis. Next in line are the extrinsic tumour suppressor mechanisms, which then are designed to remove the transformed cells that escaped the intrinsic cellular controls for abortive suicide. The immune system functions as an extrinsic tumour suppressor by detecting and eliminating tumour cells on the basis of tumour-specific antigens (Smyth et al., 2006; Swann and Smyth, 2007). The immune system is able to identify pre-cancerous and/or cancerous cells and eliminate them before these cells are able to start growing uncontrollably and result in tumour formation, a process referred to as cancer immune surveillance. The elimination phase is a part of the process termed ‘cancer immunoediting’ (Dunn et al., 2004a; Dunn et al., 2008), which is based on the concept that the immune system not only protects the host against cancer formation but also promotes cancer development. The overall process is often referred to as the ‘three Es’ of cancer immunoediting and consists of the elimination phase (cancer immuno-surveillance), equilibrium phase and escape phase (Figure 22) (Dunn et al., 2004a).
Figure 22: The ‘three Es’ of cancer immunoediting consisting of the elimination phase, equilibrium phase and escape phase (adapted and modified from Dunn et al., 2004a).
During the elimination phase, the innate and adaptive arms of the immune system work together to detect and eliminate cancer cells, which have developed as a consequence of failed intrinsic tumour suppressor mechanisms (Smyth et al., 2002). The first stage of the elimination phase mostly involves the innate immune system including macrophages and natural killer (NK) cells, which should be able to recognise developing tumours through receptor mediated recognition processes, some of these involving tumour antigen presentation (Smyth et al., 2006). Once the immune cells are activated, they then go on to utilise cytotoxic effector mechanisms in order to kill and eliminate the transformed cells or cells undergoing malignant transformation. The immune cells secrete interferons (IFNs), which control tumour growth and amplify immune responses (Smyth et al., 2006). Initially, the IFN\(\gamma\) that is released at the tumour site induces production of chemokines. These chemokines recruit cells like macrophages that produce interleukin-12 (IL-12) and NK cells that produce even more IFN\(\gamma\) at the tumour site (Bancroft et al., 1991). IFN\(\gamma\)-activated macrophages can induce apoptosis in cancer cells by releasing products such as ROS (Schreiber et al., 1983) and activated NK cells induce cancer cell death by triggering TRAIL- or perforin-dependent pathways (Smyth et al., 2001; Takeda et al., 2001; Hayakawa et al., 2002). The adaptive arm of the immune system is also an important part of the elimination phase, as effective cancer immunosurveillance responses require the additional expression of tumour antigens, which are capable of activating naïve lymphocytes into effector CD4\(^+\) and CD8\(^+\) T cells (Schreiber et al., 2011). Here, tumour-infiltrating lymphocytes (TILs) have been associated with patient survival, as increased number of TILs correlate with improved clinical outcome (Dunn et al., 2012), and they represent up to 40% of immune cells within tumours (Schreiber et al., 2011). Tumour infiltration by IFN\(\gamma\) producing Th1 CD4\(^+\) T cells and CD8\(^+\) T cells has been demonstrated in many different cancers (Mihm et al., 1996; Sato et al., 2005; Ohtani, 2007; Schreiber et al., 2011). Furthermore, it has been shown in several studies that TILs were able to lyse autologous tumour cells (Sawamura et al., 1989). Therefore, coordinated and balanced activation of both innate and adaptive immunity is necessary for tumour elimination, and if the tumour is successfully eliminated then this phase marks the endpoint of the cancer immunoediting process.

The next step of the immunoediting process is the equilibrium phase. Transformed (malignant) cells enter this phase if they are not totally eliminated during the first stage of the three E’s (Dunn et al., 2004a; Dunn et al., 2004b). This is a
temporary state in which cancer cells can remain dormant or go on to evolve by accumulating additional, more aggressive and resistant features, which are similar to CSC characteristics. The equilibrium phase is very variable in duration, it can be relatively short or long, and differs extensively with each cancer type. Once the cells in the equilibrium phase have acquired enough changes, they then enter the last, escape phase, where they are no longer detected by the immune system (Smyth et al., 2006; Swann and Smyth, 2007). Those cells, which survived the immunoediting process and have been ‘sculpted’ by the immune system, are now able to proliferate uncontrollably, form tumours and eventually metastasise.

The immune system is generally very effective in suppressing cellular transformation and cancer growth by means of the diverse intrinsic and extrinsic mechanisms as described above, resulting in cancer occurrence less than once in a lifetime, on average. This is the case even though there are many cells in the body that exist as potential targets for mutational and genetic changes or that have been mutated, and an active immune system eliminates them. Despite this, cancers do arise in immunocompetent individuals, suggesting that clonally derived populations of cells are able to escape the immune system. These cells, which emerge from the selective pressures of the immune system, are resistant to immune-induced killing due to reduced immunogenicity. These cancer cells often also have stem cell features. Furthermore and importantly, these cells are also often resistant to many of the currently used anti-cancer therapies.

2.8.1 Tryptophan Metabolism and Indoleamine 2,3-Dioxygenase
As discussed above, immune escape is the terminal stage of the immunoediting process and may be a crucial modifier of clinical outcomes, affecting tumour dormancy versus progression and permitting invasion and metastasis, as well as having an impact on the therapeutic response (Prendergast, 2008). A very interesting finding observed in studies of immune escape and metabolic alterations, which are the emerging hallmarks of cancer as described by Hanahan and Weinberg (2011), is that the microenvironmental metabolism of the essential amino acid tryptophan, catalysed by the enzyme indoleamine 2,3-dioxygenase (IDO), has an important role in mediating immune tolerance to antigens (Muller et al., 2005; Muller and Prendergast, 2007; Munn and Mellor, 2007). IDO is a haem-containing, single-chain oxidoreductase that converts Trp to N’-formyl-kynurenine by catalysing the first and rate-limiting step in the biosynthesis
of the central metabolic regulator nicotinamide adenine dinucleotide (NAD\(^+\)) (Figure 23). The expression and activity profiles of IDO are different from those of tryptophan dioxygenase (TDO), which is mainly a liver enzyme that catalyses the same reaction as IDO and maintains Trp balance in response to dietary uptake. IDO is, on the other hand, expressed in different tissues, with particularly high levels found in the epididymis, the placenta of pregnant females and across large mucosal surfaces such as the gut and lung epithelia. IDO activity usually occurs at sites of inflammation and is usually induced by IFN\(\gamma\) (Muller and Scherle, 2006).

Figure 23: Simplified schematic diagram of the tryptophan metabolic pathway in mammals. The initial and rate-limiting step is catalysed by TDO in the liver or by IDO in extrahepatic tissue. L-Trp is catabolised after IDO activation by oxidative cleavage to \(N'\)-formyl-kynurenine, which is rapidly converted to kynurenine and formic acid. Depending on the cell type, kynurenine can be further metabolised to various breakdown products, which can exert either immunological, antioxidant or neurological activities (adapted from Thomas and Stocker, 1999; Grohmann \textit{et al.}, 2003; Löb and Königsrainer, 2008).
Since excess Trp is degraded by the liver enzyme TDO and NAD$^+$ levels are maintained by the salvage mechanism, the significance of IDO was questioned. Munn and Mellor discovered that IDO suppresses T-cell activation in allogeneic pregnancy, initially, and in this way modulates immunity (Munn et al., 1998; Mellor and Munn, 2004). Several studies have indicated that T cells are preferentially sensitive to IDO activation, meaning that when starved for Trp, T cells cannot divide and hence cannot become activated by an antigen presented to them. Moreover, T cells are sensitive to kynurenine and other downstream catabolites of the IDO pathway (Fallarino et al., 2002). This together with Trp starvation seems to be important for induction of T-regulatory (T$_{reg}$) cells and immune suppression (Fallarino et al., 2006; Munn and Mellor, 2007). A crucial connection between cancer and IDO stems from the demonstration that IDO is chronically activated in many patients with cancer (Uyttenhove et al., 2003), such as the ovarian and colorectal neoplastic disease (Okamoto et al., 2005; Brandacher et al., 2006). Among the first observations that indicated a role of IDO in cancer, although not obvious at that stage, was elevation of Trp metabolites in the urine of bladder cancer patients (Boyland and Williams, 1955). This observation was also seen later in breast and prostate cancer, Hodgkin’s lymphoma and leukaemia patients (Ambanelli et al., 1962; Ivanova, 1964; Rose, 1967; Wolf et al., 1968). IDO activation has also been correlated with a more extensive neoplastic disease (Ino et al., 2006; Takao et al., 2007; Pan et al., 2008).

Given the information above, it is clear that IDO plays an important role in cancer cell immunity. It participates in both passive and active defense of cancer cells from the immune system by starving effector T cells for Trp, jeopardising their division and preventing their activation by an antigen presented to them, and by inducing naïve T cells into T$_{reg}$ cells, which then protect cancer cells from the immune system, respectively. Therefore, inhibition of IDO has been considered as a potential cancer treatment strategy. IDO inhibitors, such as brassinin or 1-methyl tryptophan (1MT), are being investigated as anti-cancer drugs (Gaspari et al., 2006; Hou et al., 2007), with D-1MT (one of two stereoisomers of 1MT) undergoing clinical trials (Katz et al., 2008). Recent study by Opitz and colleagues showed that TDO also plays a role in mediating anti-tumour immune responses (Opitz et al., 2011). They demonstrated that TDO was constitutively expressed in glioma cells and that Trp degradation by TDO into kynurenine lead to the activation of the aryl hydrocarbon receptor (AHR), which resulted in suppression of anti-tumour immune responses and promotion of tumour-cell
survival. Further, endogenous production of kynurenine, kynurenine being an endogenous ligand of the AHR (Platten et al., 2012), in glioma cells was enough to activate AHR, as knocking-down TDO resulted in a decreased expression of AHR-regulated genes (Opitz et al., 2011). Also in vivo experiments showed that induced knockdown of AHR in glioma cells inhibited tumour growth in immunocompromised mice. This study also demonstrated that TDO-derived kynurenine activated the AHR in other cancers apart from glioma including B-cell lymphoma, Ewing sarcoma, bladder, cervix, colorectal, lung and ovarian carcinomas (Opitz et al., 2011). Another group recently showed TDO expression in bladder carcinoma, melanoma and hepatocarcinoma (Pilotte et al., 2012). They demonstrated that TDO inhibition in vivo resulted in tumour control in immunocompetent mice, however, unlike in the study by Opitz et al., not in immunodeficient mice. These results indicate that, as well as IDO, TDO may be a novel target for anti-cancer therapy and recently, a promising TDO inhibitor with high selectivity, and good oral bioavailability has been developed (Dolusic et al., 2011).

2.9 Resistance of CSCs to Treatment

The major problem with CSCs is that, similarly to normal stem cells, they feature properties which make them resistant not only to the immune system as discussed above, but, importantly too, also to current cancer therapies such as chemotherapy and radiation therapy, thereby promoting their survival (Frank et al., 2003; Frank et al., 2005; Bao et al., 2006; Hambardzumyan et al., 2006). Some of these characteristics were discussed in the CSC chapter. It has been implied that during cancer therapy, a low percentage of cells with stem-like features (CSCs) escapes the killing efficacy of established anti-cancer drugs. These cells, which have acquired an aggressive phenotype, are able to give rise to secondary tumours, which become highly resistant to further treatment after selection during the initial therapy (Neuzil et al., 2007).

As described above, cancer often acquires resistance to treatment in response to non-lethal exposure to chemotherapy and/or radiotherapy and even tumour-targeted agents, which eliminates the bulk of the tumour (fast-proliferating cells) leaving slow-growing cells with stem-like features (Blagosklonny, 2005). In this way, resistant CSCs are ‘naturally’ selected and propagated (Morrison et al., 2011). Increased resistance has been shown for many types of CSCs, including leukaemic (Essers and Trumpp, 2010), brain (Bao et al., 2006; Liu et al., 2006; Hambardzumyan et al., 2006; Dirks 2010),
pancreatic (Lonardo et al., 2010), breast (McDermott and Wicha, 2010), melanoma (Frank et al., 2003, 2005), as well as colon CSCs (Boman and Huang, 2008). Figure 24 documents the characteristics of treatment-resistant CSCs. Resistance to DNA damage has been implicated in one study showing that radiation caused equal levels of damage to all cancer cells, although CSCs were able to repair this damage more rapidly (Eyler and Rich, 2008). It has been shown that checkpoint kinases 1/2 (Chk 1/2 kinases) have higher basal and inducible activities in CSCs, indicating their supporting role in these cells (Bao et al., 2006). Also, telomerase function has been shown to be downregulated in brain CSCs and several drugs that interfere with this function are already in clinical trials, such as arsenic oxide, GRN163L, and certain vaccines (Miller et al., 2002; Vonderheide et al., 2004; Danet-Desnoyers et al., 2005; Dikmen et al., 2005; Estey et al., 2006).

Figure 24: Schematic diagram representing the mechanisms, which lead to CSC resistance to commonly used treatments such as chemotherapy and radiation therapy (Morrison et al., 2011).

In a study performed by Liu et al. (2006), the gene expression profile was examined in CD133+ glioblastoma cell lines prepared from cancer patients. The authors found that the CD133+ cells expressed much higher levels of the neural precursor markers CD90, CD44, CXCR4, nestin, MsiI and MELK in comparison with autologous CD133- cells. They also reported that CD133+ cells express higher levels (by a factor of up to 300) of the anti-apoptotic proteins Bcl-2, Bcl-xL, FLIP, c-IAP2, XIAP, NAIP and
survivin, whereas the expression of the pro-apoptotic protein Bax was downregulated by about 5-fold when compared to their CD133− counterparts (Table 6). The CD133+ cells were also found to be resistant to the drugs etoposide, paclitaxel, temozolomide and carboplatin, while their autologous CD133− cells were susceptible. Due to the fact that CD133+ cells express high levels of the caspase-8 inhibitor FLIP, they can be expected to be resistant to the induction of the extrinsic, death receptor-mediated apoptotic signalling pathway. Furthermore, it was demonstrated that expression of CD133 was much higher in the recurring glioblastoma multiforme than in newly diagnosed gliomas, indicating that glioblastoma stem cells were able to survive treatment and promote repeated tumourigenesis (Liu et al., 2006).

Table 6: Differences in the mRNA expression profile in CD133+ cells compared to autologous CD133− cells (Liu et al., 2006).

<table>
<thead>
<tr>
<th>Section</th>
<th>Gene name</th>
<th>No. 66 CD133−</th>
<th>No. 66 CD133+</th>
<th>No. 377 CD133−</th>
<th>No. 377 CD133+</th>
<th>No. 1049 CD133−</th>
<th>No. 1049 CD133+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CD90</td>
<td>15.6 ± 0.66</td>
<td>12.8 ± 0.94</td>
<td>13.5 ± 0.75</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>CD44</td>
<td>5.7 ± 0.48</td>
<td>2.5 ± 0.22</td>
<td>2.8 ± 0.19</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>CXCR4</td>
<td>337.8 ± 29.2</td>
<td>251.5 ± 22.1</td>
<td>264.9 ± 22.9</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Nestin</td>
<td>21.4 ± 1.25</td>
<td>23.2 ± 1.65</td>
<td>22.1 ± 1.54</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>MSI</td>
<td>84 ± 7.6</td>
<td>75.4 ± 7.03</td>
<td>53.5 ± 6.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MELK</td>
<td>1351 ± 95.8</td>
<td>467.7 ± 40.5</td>
<td>514.6 ± 45.6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>GLI-1</td>
<td>46 ± 3.8</td>
<td>43 ± 4.5</td>
<td>49 ± 5.9</td>
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<tr>
<td></td>
<td>PTCH</td>
<td>16 ± 1.48</td>
<td>13.5 ± 0.85</td>
<td>14.3 ± 1.24</td>
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<td></td>
<td></td>
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<tr>
<td>B</td>
<td>MGMT</td>
<td>32.4 ± 2.5</td>
<td>34.7 ± 2.9</td>
<td>56.3 ± 4.2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCRP1</td>
<td>6.5 ± 0.43</td>
<td>4.3 ± 0.25</td>
<td>4.8 ± 0.24</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>SIRT1</td>
<td>4.9 ± 0.34</td>
<td>4.2 ± 0.26</td>
<td>5.4 ± 0.29</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>FLIP</td>
<td>294 ± 25.5</td>
<td>157.6 ± 14.2</td>
<td>145.6 ± 13.7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>BCL-2</td>
<td>13.9 ± 0.95</td>
<td>4.9 ± 0.54</td>
<td>3.8 ± 0.54</td>
<td></td>
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<tr>
<td></td>
<td>BCL-XL</td>
<td>5.6 ± 0.39</td>
<td>3.2 ± 0.16</td>
<td>2.5 ± 0.14</td>
<td></td>
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<tr>
<td></td>
<td>cIAP1</td>
<td>39.0 ± 3.5</td>
<td>4.3 ± 0.53</td>
<td>5.6 ± 0.65</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cIAP2</td>
<td>3 ± 0.25</td>
<td>1.9 ± 0.12</td>
<td>1.7 ± 0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>XIAP</td>
<td>21.9 ± 2.2</td>
<td>9.7 ± 0.68</td>
<td>10.3 ± 0.91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAIP</td>
<td>12.1 ± 0.75</td>
<td>6.4 ± 0.43</td>
<td>4.5 ± 0.62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Survivin</td>
<td>1.6 ± 0.08</td>
<td>2.3 ± 0.18</td>
<td>2.4 ± 0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAX</td>
<td>0.33 ± 0.03</td>
<td>0.49 ± 0.06</td>
<td>0.21 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total RNA was extracted from both CD133+ and CD133− fractions of three primary cultured cells (No. 66, 377 and 1049) following FACS sorting. mRNA expression was measured using real-time qPCR, and the relative mRNA expression of different genes in CD133+ cells was presented as the fold increase compared to that of autologous CD133− cells. Data is presented as mean ± SD of three independent experiments.
A number of reports have also documented increased membrane transporter activity with upregulation of the ATPase pump ABCG5 in CD133+ cells, as well as in the progenitor cells of human epidermal melanocytes and a subpopulation of malignant melanoma cells (Frank et al., 2003). A recent study revealed that CD133+/ABCG5+ melanoma cells were resistant to treatment with doxorubicin, and melanoma tissue isolated from patients expressed high levels of both CD133 and ABCG5 (Frank et al., 2005). The combined use of chemotherapeutic drugs and inhibitors targeting ABC transporters could be used to selectively and more efficiently eradicate CSCs (Dean et al., 2005). Specific inhibitors of the ABC protein, such as ABC1, are in clinical use, and some of these inhibitors also affect ABCG5 (Robey et al., 2004). However, the use of such inhibitors has to be very strictly monitored, since they are likely to cause toxicity of normal stem cells and since they play an important role in maintaining the blood-brain barrier (Lou and Dean, 2007). The tumour microenvironment has also been implicated in CSC resistance to treatment due to increased production of free-radical scavengers and protection by a hypoxic niche (Morrison et al., 2011).

Not only chemotherapy and radiation therapy, but even many novel tumour-targeted agents affect only the rapidly proliferating cancer cells, leaving CSCs untouched and resistant. An example of this is imatinib (Gleevec; Novartis) targeting and inhibiting the BCR-ABL kinase, which acts as a molecular switch that promotes proliferation and differentiation of multipotent progenitors in CML (Era et al., 2002). BCR-ABL is needed for the survival of proliferating progenitor cells, but not the quiescent CML stem cells (Bedi et al., 1993; Holyoake et al., 1999; Graham et al., 2002). Hence, even though the mutations are thought to accumulate in CSCs, their functional effects are mostly revealed further downstream in the tumour hierarchy, which leads to the neoplastic proliferation of primitive progenitors. In the light of this, imatinib eradicates proliferating, committed leukaemia progenitors, but not primitive, quiescent CSCs (Zhou et al., 2009). Also, alterations in Ikaros family zinc finger 1 (IKZF1) are thought to synergise with BCR-ABL to induce lymphoblastic leukaemia and contribute to drug resistance and disease progression (Mullighan et al., 2008).

It is quite obvious that current cancer treatments are not effective for all cancer cells with the prospect that CSCs are able to evade therapy. It is, therefore, necessary to generate novel therapeutic strategies in order to eradicate these resistant cells and in that way prevent cancer recurrence. Such treatment must target CSCs as well as their microenvironmental niche by targeting the associated signalling pathways (Fan et al.,
2006; Clement et al., 2007) and the cell cycle machinery (Bao et al., 2006), using siRNA for example (Thomas et al., 2005), as well as by inhibiting angiogenesis (Folkman, 2002).

**2.10 Drugs Efficient against CSCs**

Since current cancer therapies usually fail to cure cancer completely, it is of paramount importance to find new strategies, which would especially target CSCs. One of the exceptions to the current unsuccessful treatments is surgery, if it is applicable, performed at the early stage of the disease, such that all (or majority) of the diseased tissue is removed. This approach is often impossible to apply successfully. The novel potential strategies to eliminate CSCs include the blockage of essential self-renewal signalling pathways (Fan et al., 2006; Clement et al., 2007), the inhibition of the survival mechanisms and the cell cycle machinery (Bao et al., 2006), the induction of apoptosis, as well as targeting CSC surface markers through antibody-based cytotoxic approaches (Zhou et al., 2009). Induction of tumour cell differentiation, which can potentially be achieved by inhibiting developmental pathways or epigenetic programmes (Korkaya and Wicha, 2007; Zhou et al., 2009), and inhibition of angiogenesis, which depletes oxygen and nutrient supplies (Folkman, 2002), are other potential approaches. Also, it is possible that CSCs depend on their niche to maintain their ‘identity’, such that targeting the niche could be an intriguing strategy to indirectly suppress the proliferation and induce the differentiation of CSCs (Zhou et al., 2009) (Figure 25).
Figure 25: Current therapeutic strategies. These include targeting candidate CSCs and their microenvironmental niche, which contributes to their self-renewal. A novel strategy could involve targeting the ROS status of these cells and altering their intracellular milieu in order to induce apoptosis and suppress proliferation (Tang et al., 2007).

2.10.1 Current Trends in Design of Drugs Selective for CSCs
Recently, there has been much of interest and numerous studies focusing on the development of drugs effective against CSCs. There are several different approaches for targeting these resistant cells and they have been described in the previous chapter.

One of the different strategies to inhibiting CSC growth is to target pathways that are necessary for cell survival, such as the HH, Notch and Wnt signalling. One study has demonstrated that blocking the Notch pathway (as Notch is also highly expressed in CSCs) by inhibiting $\gamma$-secretase resulted in inhibition of expression of Hes1 and in induction of apoptosis in medulloblastomas (Georgia et al., 2006). Furthermore,
the more differentiated Notch-inhibited cell lines, while remaining viable, did not form tumours when injected into NOD/SCID mice. Another study showed that inhibition of the Notch pathway in breast cancer with increased Notch activity reduced tumour growth (Pece et al., 2004). Similarly, inhibition of γ-secretase with GSI-18 ([11-endo]-N-(5,6,7,8,9,10-hexahydro-6,9-methanobenzo[a][8]annulen-11-yl)-thiophene-2-sulfonamide) attenuated growth of embryonal brain tumours, as well as blocked Notch signalling, which resulted in a decrease in the stem cell population (Fan et al., 2006). Inhibition of the HH pathway using Smo shRNA decreased the proliferative rate of CD133+ cells and their ability to form gliomaspheres and increased survival of mice with glioblastoma tumours.

Cyclopamine, a pharmacological inhibitor of the HH pathway, was able to reduce the number of spheres, leading to the proposal that it may be possible to use combinatorial treatment to kill CSCs (Clement et al., 2007). CD133+ cells that are normally resistant to radiotherapy became sensitised to radiation-induced killing upon inhibiting the checkpoint kinases Chk1 and Chk2, thereby promoting brain tumour recurrence (Bao et al., 2006). Several extracellular Wnt inhibitors, such as the secreted Frizzled-related proteins (SFRPs) and the Dickkopf proteins, which inhibit Wnt signalling via its receptors, have been discovered (Kawano and Kypta, 2003) and are of potential use in resistant CSC elimination. The cell cycle inhibitor temsirolimus (CCI-779) is a rapamycin ester, which was shown to be effective in inhibiting mTOR in several breast cancer cell lines with increased Akt activity (Yu et al., 2001). One study also reported that temsirolimus provoked anti-tumour responses in a xenograft model of melanoma and that these anti-tumour responses were associated with induced apoptosis and decreased proliferation and angiogenesis (Frost et al., 2004). The tumour suppressor gene TP53 also seems to have a crucial role in tumourigenesis and stem cell self-renewal. Small molecule inhibitors (nutlins, spiro-oxindales) of MDM2-p53 interactions selectively enhanced the cytotoxicity of chemotherapeutic agents in acute myelogenous leukaemia (AML) blasts but not in normal haematopoietic progenitors (Vassilev, 2004; Ding et al., 2006).

Another approach to the treatment of resistant cancer cells is to target ABC transporters, which are often upregulated in CSCs. Specific inhibitors of the ABC proteins, such as ABC1, are in clinical use and some of these inhibitors also affect ABCG2 (Robey et al., 2004). The combined use of chemotherapeutic drugs and inhibitors of ABC transporters could be used to selectively and more efficiently
eradicate CSCs (Dean et al., 2005). However, the use of such inhibitors has to be very strictly monitored due to the fact that they are likely to cause toxicity of normal stem cells and since they play an important role in maintaining the blood-brain barrier (Lou and Dean, 2007).

Another approach to treatment is targeting CSC surface markers through antibody-based cytotoxic approaches. Jin et al. (2006) have explored the possibility of targeting CD44 by an activating monoclonal antibody (H90). Administering H90 to immunodeficient mice, which were transplanted with human AML, significantly reduced leukaemic re-population. Moreover, the demise of the leukaemic population and the immense decrease in the number of CD34^+CD38^- cells in serially transplanted mice suggested that it was the leukaemia-initiating stem cells, which were the target of this therapeutic approach (Jin et al., 2006). The cell surface marker CD44 is also expressed on a variety of CSCs including breast, colon, prostate, head, neck, and pancreatic tumours, suggesting that a similar approach may prove effective in treating these malignancies. Another recent study also demonstrated the potential therapeutic use of targeting the cell surface marker CD133, to direct therapy specifically towards CSCs (Wang et al., 2011).

Inducing terminal differentiation of CSCs, which will result in depletion of those cells with self-renewal potential, is another strategy in targeting resistant CSCs, although this approach has so far had a variable success (Spira and Carducci, 2003; Sell, 2004). All-trans-retinoic acid (ATRA - tretinoin) and sodium phenylbutyrate have been used for the treatment of haematologic malignancies that exhibit blocks in differentiation (Bruserud and Gjertsen, 2000). Differentiation induction therapy with ATRA is now readily applied in the treatment of acute promyelocytic leukaemia (APL) (Tallman MS, 1996; Lo Coco et al., 1998; He et al., 1999; Kogan and Bishop, 1999), and its use for other AMLs is being explored. ATRA belongs to the retinoid family of vitamin A derivatives (Gillis et al., 1995; Tallman MS, 1996; Lo Coco et al., 1998; He et al., 1999; Kogan and Bishop, 1999). Several in vitro studies have demonstrated that arsenic trioxide (ATO) is effective against APL cells and therefore should be considered as a promising therapeutic agent with limited toxicity (Niu et al., 1999). At low doses ATO was able to induce partial differentiation, and at higher doses it triggered apoptosis in APL cells (Chen et al., 1997). Combination of ATO with cyclic AMP (cAMP) or the granulocyte-macrophage colony-stimulating factor (GM-CSF) resulted in terminal differentiation in APL cells (Muto et al., 2001; Zhu et al., 2002). It seems that the
molecular mechanisms of the differentiation effect of both ATO and ATRA include ligand binding to promyelocytic leukaemia-retinoic acid receptor α (PML-RARα), which causes degradation of fusion proteins and alters transcription regulation (Kogan and Bishop, 1999), allowing for the APL cells to respond to therapy (Shackelford et al., 2006). Other anti-cancer agents that have been found to induce differentiation in AML cell lines and in native AML blasts include cytarabine, daunorubicin and 6-thioguanine (Hassan and Rees, 1989a; Hassan and Rees, 1989b; Tawhid and Rees, 1990; Bloch, 1993). Monosaccharide butyrate derivates have also been shown to induce differentiation in AML cells (Santini et al., 1998). Several in vitro studies have demonstrated that the differentiation induction effects of ATRA can be enhanced by several other agents (Fenton et al., 1999; Saito et al., 1999), including the drug clofibric acid (Fenton et al., 1999).

An interesting anti-cancer agent, which has been widely studied and is in clinical trials, is the sesquiterpene lactone parthenolide, a natural product isolated from medicinal plants including Tanacetum parthenium (feverfew). Parthenolide has been initially found to inhibit the transcription factor NFκB (Bork et al., 1997), by way of inhibiting activation of its inhibitory components (Hehner et al., 1998). Parthenolide has been shown to suppress proliferation and induce apoptosis of leukaemia stem cells (Guzman et al., 2005a,b, 2007). Therefore, a number of sesquiterpene lactone analogues have been synthesised and tested as anti-cancer drugs (Ghantous et al., 2010). Recently, it has been reported that parthenolide also targets breast and prostate CSCs (Liu et al., 2008; Zhou et al., 2008; Kawasaki et al., 2009). Salinomycin is another recently discovered anti-CSC drug, which is efficient against breast CSCs and has a potential clinical application (Gupta et al., 2009; Rowan, 2009). This agent was, for example, some 100-fold more efficient in lowering the proportion of CSCs in the cancer cell population than the established anti-cancer agent paclitaxel. Metformin, an oral anti-diabetic drug (Crandall et al., 2008), has been recently reported to target breast CSCs and, when combined with doxorubicin, prevent growth of tumours as well as their remission (Hirsch et al., 2009). Due to the fact that metformin acts by interfering with the energy metabolism of cells, it may inhibit self-maintenance of mitotically competent cells acting as a caloric restriction mimetic (Martin-Castillo et al., 2010; Vazquez-Martin et al., 2010; Nguyen et al., 2010). Another anti-cancer drug that has been used for treatment of Herceptin-resistant, metastatic breast cancer cells is lapatinib (Burris et al., 2005). This dual receptor tyrosine kinase inhibitor (suppressing the activation of
HER2/erbB2 and EGFR) has been suggested to suppress the growth of CSCs in the context of HER2-high breast and lung cancers (Magnifico et al., 2009; Korkaya and Wicha, 2009; Diaz et al., 2010).

Targeting the tumour microenvironment niche is another possible approach to treating resistant cancer cells. CSCs are able to remain dormant in these niches or slowly develop over decades. It seems that CSCs may preferentially localise to environments that favour proliferation, such as vascular niches (Sipkins et al., 2005; Calabrese et al., 2007; Hambardzumyan et al., 2008), or even promote the formation of their own niche (Bao et al., 2006). This was indicated in a study that showed that haematopoietic stem cells and possibly leukaemic stem cells localised to endothelial niches (Kiel et al., 2005). This was also shown in another study, which proposed that neural stem cells localised to vascular niches (Palmer et al., 2000; Capela and Temple, 2002; Louissaint et al., 2002) and that brain tumour stem cells reside in a perivascular niche (Calabrese et al., 2007; Hambardzumyan et al., 2008). Furthermore, glioma stem cells were shown to promote tumour angiogenesis (Bao et al., 2006), suggesting that these cells may also take part in the formation of their own niche. One notion suggests that CSCs are held in check by a niche-stem cell interaction (Zhou et al., 2009), which needs to be interrupted in order to eradicate CSCs. Some agents that target CSC markers may also work by affecting the niche. For example one study demonstrated that an activated mAb specific for the adhesion molecule CD44 was shown to eradicate human AML leukaemic stem cells in vivo by blocking the trafficking of leukaemic stem cells to supportive microenvironments, and by altering their ‘stem cell’ fate through differentiation (Jin et al., 2006). This study also demonstrated that AML leukaemic stem cells require the interaction with a niche to maintain their stem cell properties. This type of treatment may be applicable to other types of CSCs. Targeting the vascular niche of CSCs with anti-angiogenic agents may also be a plausible treatment of resistant cancer cells (Zhou et al., 2009). Several anti-angiogenic agents including for example thalidomide, bevacizumab, temesirilimus and everolimus have been clinically approved for treatment of different types of cancers such as renal cell carcinoma, colorectal and breast cancer and multiple myeloma (Fan et al., 2012). However, the effect of these agents on the vascular CSC niche needs to be further studied and determined. Nevertheless, the association between CSCs and the vascular niche indicates the possibility of combining agents that target CSCs with anti-angiogenic therapy. In a comparable way other CSC niches could also be specifically targeted.
Taken together, there are studies focusing on the design of novel drugs showing effectiveness against CSCs, which may be anticipated to have a significant anti-cancer effect, minimising recurrence of the pathology. Some of these agents have shown promising results, some have even entered clinical trials and many more are being studied.

2.10.2 Mitocans as Promising Agents
Mitochondria have recently emerged as an intriguing target for anti-cancer drugs, active against the vast majority if not all types of tumours (Ralph et al., 2010a, b). This has been corroborated by recent findings that tumours differ in the level of expression of a high number of genes and mutations even amongst patients with the same type of tumour as has been documented for pancreatic cancer and glioblastoma multiforme (Jones et al., 2008; Parsons et al., 2008). Further, differences in the genomic profile of individual regions of the same tumour have been reported (Gerlinger et al., 2012). This suggests that suppressing cancer by targeting a single gene or a single pathway that may alter amongst cancer patients and that can be subject to mutations poses significant challenge. It is therefore imperative to search for a target that is invariant and whose exploitation may present a general strategy for efficient treatment across the landscape of neoplastic pathologies. Mitochondria may present such a target.

Mitochondria play a key role in the intrinsic apoptotic pathway. Also, the aberrant bioenergetic metabolism of mitochondria in malignant cells is important and ought to be taken into consideration (Koppenol et al., 2011; Ward and Thompson, 2012). Therefore, novel agents that target mitochondria specifically in order to induce cancer cell death have been the focus of many recent studies, with some ‘old’ compounds being ‘re-discovered’ for their propensity to destabilise mitochondria and kill cancer cells. Correspondingly and with an indisputable involvement in the molecular mechanism of the mitochondria-targeting anti-cancer agents, the Warburg's hypothesis published in the 1920s (Warburg, 1956) has been recently experiencing a renaissance (Cairns et al., 2011; Hanahan and Weinberg, 2000, 2011; Koppenol et al., 2011; Vander Heiden et al., 2009).

Mitocans (an acronym for ‘mitochondria and cancer’) are a class of anti-cancer drugs that act by way of mitochondrial destabilisation. Many of these drugs have been known for a long time and have now been classified into several classes based on their molecular mode of action by Neuzil et al (2012). Table 7 provides several examples
epitomising the individual classes of mitocans, in particular those that are perceived as potentially clinically relevant anti-cancer agents. The classification is based on the site of action of the individual agents from the surface of the mitochondrial outer membrane (MOM) to the mitochondrial matrix. Figure 26 illustrates the molecular targets of individual classes of mitocans. These compounds have been proposed to hold a considerable promise as future anti-cancer drugs (Neuzil et al., 2012).
Table 7: Classification of mitocans and examples of compounds in individual classes (Neuzil et al., 2012).

<table>
<thead>
<tr>
<th>Class number</th>
<th>Type</th>
<th>Examples</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexokinase inhibitors</td>
<td>3BP, 2DG</td>
<td>Ko et al., 2004; Mathupala et al., 2006; Simons et al., 2007; Chen et al., 2009; Ben Sahra et al., 2010</td>
</tr>
<tr>
<td>2</td>
<td>Compounds targeting Bcl-2 family proteins</td>
<td>Gossypol, ABT-737, antimycin A, α-TOS</td>
<td>Tzung et al., 2001; Pelican et al., 2003; van Delft et al., 2006; Shiau et al., 2006; Kang et al., 2009</td>
</tr>
<tr>
<td>3</td>
<td>Thiol redox inhibitors</td>
<td>Isothiocyanates, arsenic trioxide</td>
<td>Xu and Thornalley, 2001; Miller, 2002; Pelican et al., 2003; Trachootham et al., 2006</td>
</tr>
<tr>
<td>4</td>
<td>VDAC/ANT targeting drugs</td>
<td>Lonidamide, arsenites, steroid analogues such as CD437</td>
<td>Belzacq et al., 2001; Don et al., 2003</td>
</tr>
<tr>
<td>5</td>
<td>Electron redox chain targeting drugs</td>
<td>α-TOS, MitoVES, tamoxifen, adaphostin, 3BP</td>
<td>Moreira et al., 2006; Le et al., 2007; Dong et al., 2008, 2009, 2011a,b; Pereira da Silva et al., 2009; Rohlina et al., 2011</td>
</tr>
<tr>
<td>6</td>
<td>Lipophilic cations targeting inner membrane</td>
<td>Rhodamine-123, F16, (KLAKKLAK)₂</td>
<td>Johnson et al., 1980; Bernal et al., 1983; Lampidis et al., 1983; Ellerby et al., 1999; Fantin et al., 2002</td>
</tr>
<tr>
<td>7</td>
<td>Drugs targeting the tricarboxylic acid cycle</td>
<td>DCA, 3BP</td>
<td>Bonnet et al., 2007; Dell’Antone, 2009; Pereira da Silva et al., 2009</td>
</tr>
<tr>
<td>8</td>
<td>Drugs targeting mtDNA</td>
<td>Vitamin K3, fialuridine, 1-methyl-4-phenyl-pyridinium, MitoVES</td>
<td>Lewis et al., 1996; Miyako et al., 1997; Umeda et al., 2000; Sasaki et al., 2008; Truksa et al., unpublished</td>
</tr>
<tr>
<td>9</td>
<td>Drugs targeting other (unknown) sites</td>
<td>Betulinic acid</td>
<td>Fulda et al., 1998</td>
</tr>
</tbody>
</table>
Figure 26: Schematic illustration of the molecular targets of individual classes of mitocans. The classes of mitocans consist of the following, viewed from the outside of mitochondria towards the matrix. Class 1: hexokinase inhibitors; Class 2: Bcl-2 homology domain 3 (BH3) mimetics and compounds that impair the function of the anti-apoptotic Bcl-2 family proteins; Class 3: thiol redox inhibitors; Class 4: VDAC- and ANT-targeting agents; Class 5: compounds targeting the mitochondrial electron transport chain (ETC); Class 6: hydrophobic cations targeting the mitochondrial inner membrane (MIM); Class 7: compounds that affect the tricarboxylic acid cycle (TCA), and Class 8: agents targeting mtDNA. Class 9 (not shown) includes agents acting on mitochondria, whose molecular target has not been described so far (Neuzil et al., 2012).

One of the mitocans that has been extensively studied is a compound from the group of vitamin E analogues, epitomised by the redox-silent α-tocopheryl succinate (α-TOS) (Neuzil et al., 2001; Weber et al., 2002) (Figure 27). This agent acts by targeting the mitochondrial complex II (CII), thereby causing generation of high levels of ROS,
which then induce apoptosis by destabilising the MOM (Dong et al., 2008, 2009), by promoting the formation of the Bak channel in mitochondria in a FoxO-NOXA-dependent manner (Prochazka et al., 2010; Valis et al., 2011). α-TOS also acts by activating SMase giving rise to the lipid second messenger ceramide, which destabilises the mitochondrial membrane leading to apoptosis as described in Figure 28 (Neuzil et al., 2004).

![Image removed]

**Figure 27**: Structure of vitamin E (α-tocopherol; α-TOH) and its esterified analogue (α-tocopheryl succinate; α-TOS) (Birringer et al., 2003).
Figure 28: A scheme of apoptotic signalling pathways induced in response to α-TOS. α-TOS translocates to the cell, where it activates sphingomyelinase (SMase), possibly causing the destabilisation of lysosomes, giving rise to the lipid second messenger ceramide, which leads to the destabilisation of the mitochondrial membrane. α-TOS directly and/or via ceramide formation destabilises mitochondrial membrane, and the ROS generated in response to the agent may amplify this process. Mitochondrial membrane destabilisation, likely promoted by leakage by lysosomal proteases, leads to cytosolic relocalisation of pro-apoptotic factors (such as cytochrome c, Smac/Diablo or AIF) that can be regulated by Bcl-2 family proteins (including Bcl-2, Bcl-xL or Mcl-1, which can be compromised by another Bcl-2-related protein Bax, mobilized to mitochondria after cleavage of Bid to its pro-apoptotic form). Cytochrome c, Apaf-1 and pro-caspase-9 form a ternary complex, leading to the activation of the initiator caspase-9 that, in turn, results in the activation of the effector caspases. Smac/Diablo may amplify this process by suppressing the caspase inhibitory activity of inhibitor of apoptosis proteins (IAPs) family, while AIF is supposed to transmit mitochondrial destabilisation to nuclear apoptotic events (Neuzil et al., 2004).

In order to enhance the activity of α-TOS, this agent was modified by tagging it with the positively charged triphenylphosphonium (TPP⁺) group, as has been suggested for a variety of redox-active compounds (Murphy and Smith, 2007; Biassutto et al., 2010). This resulted in the generation of mitochondrially targeted vitamin E succinate.
(MitoVES) (Figure 29). The TPP$^+$ modification of the compound allows it to move across most biological membranes, targeting it to the mitochondrial inner membrane (MIM). At neutral pH and positive potential ($\Delta \Psi$), the phosphorous positive charge is delocalised on the phenyl groups. At low mitochondrial $\Delta \Psi$, the charge is localised and the compound consequently accumulates in the MIM, resulting in a considerable increase in its local concentration. This property of MitoVES can be expected to maximise its biological activity. In fact, it has been shown that MitoVES was 1-2 log more efficient in killing cancer cells than its untargeted counterpart (α-TOS), and this was paralleled by its activity on experimental cancer, including colon cancer and HER2-high breast cancer (Dong et al., 2011; Neuzil et al., unpublished). Not only did this anti-cancer agent show very high efficacy against fast-proliferating cancer cells, but it was recently demonstrated that MitoVES shows high efficacy against CSCs, represented by mammospheres as multicellular structures with enhanced level of stemness, characterised as CD44$^{\text{high}}$/CD133$^{\text{high}}$/CD24$^{\text{low}}$/Jagged-1$^{\text{high}}$ cells (Neuzil et al., unpublished).
Figure 29: The principle of mitochondrial targeting. Top part: Tagging a cationic TPP⁺ group to hydrophobic compounds, with the charge on the phosphorus delocalised on the flanking phenyl groups, causes their relatively free movement across biological membranes. Once in the mitochondrial matrix with the negative potential on the matrix face of the MIM, the TPP⁺ group anchors the compound at the matrix-MIM interface, causing an increased concentration of the agent in this compartment. This is important for enhancing the bioactivity of agents, whose target is localised close to the interface. Bottom part: Structure of mitochondrially targeted vitamin E succinate (MitoVES).

While the mechanism is not clear at this stage and it remains to be determined whether mitochondria in slow proliferating CSCs are different from those in fast-proliferating cancer cell populations or normal cells, agents like MitoVES may present a substantial promise for the development of compounds that will efficiently eradicate not only the bulk of the tumour cells but, more importantly, also the highly recalcitrant
CSCs, thereby minimising the probability of tumour remission. MitoVES, importantly, can bypass some of the mechanisms in cancer cells that make them resistant to conventional therapies (Neuzil et al, unpublished). Therefore, it seems that targeting mitochondria directly using mitocans could be useful for eradicating CSCs either alone or in combination with other approaches.

In order to comply with copyright chapters 3, 4, 5 and 6 have been removed.
Gene Microarray Analysis

Pre-processing, normalisation and Principal Components Analysis (PCA): The first component of the microarray analysis used the software package BeadStudio to extract the raw expression data. Pre-processing of microarray data was then done using the R software package Linear Models for Microarray Data (LIMMA) (1). The ‘normexp’ function was used for control background correction, quantile normalisation and log₂ transformation of the raw data (2).

To assess global expression in adherent cells and spheres, we used principle components analysis (PCA) in the ArrayTrack software environment (3). PCA uses a mathematical algorithm that reduces the dimensionality of the microarray data whilst retaining the variation in the data. It reduces the dimensionality by identifying directions, called principal components, along which the variation in the data is maximal (4). The global gene expression of adherent cells and spheres can then be plotted, making it possible to visually assess similarities and differences between samples.

Gene Set Enrichment Analysis (GSEA): GSEA was used to assess for stemness in spheres when compared to adherent cells. Haematopoietic (HSC), embryonic (ESC) and neural (NSC) stem cell gene sets were derived from an overlap of stemness genes characterized in several microarray studies (5-7) (Fig 1). To determine the enrichment of HSC, ESC and NSC gene sets in spheres, all genes expressed on the microarrays were analyzed using GSEA analysis (8). GSEA was used to assess for stemness by determining whether HSC, ESC and NSC gene sets were significantly enriched in spheres when compared to adherent cells. GSEA was also used to characterize metabolic and signaling pathways enriched in spheres. Pathway analysis of the expression data was performed using the GSEA implemented in GSEA java desktop application version 2.0 and MsigDB (Molecular Signature Database) version 2.0. Pathways derived from the Molecular Signatures Database (MSigDB) on the GSEA website were used to assess enrichment in both adherent cells and spheres. GSEA was performed using a total of 880 gene sets containing genes whose products are involved in specific metabolic and signaling pathways. For this study the top 10 up- and down-regulated pathways in spheres with a significant enrichment score ($p<0.05$) and false discovery rate (FDR) ($q<0.05$) were considered for further interpretation.
The statistical significance (p-value) of each gene set was estimated by using phenotype-based permutation analysis using 1,000 random permutations. We accepted gene sets with a significant enrichment score (ES) \( p < 0.05 \) adjusted for multiple comparisons using the FDR of \( q < 0.05 \) or very close to \( q < 0.05 \) for further interpretation. The FDR is a quantity that describes, for a set of tests that are called significant at or above a given level, what proportions are likely to be false positives (9). Each of the enriched gene sets contained leading edge genes (LEGs). These genes contribute most to the enrichment score of the gene set and therefore are likely to participate in a biological response. We used the default setting signal-to-noise (SN) ranking metric in GSEA to rank genes. In this case, LEGs of enriched gene sets are those whose expression correlates with a given phenotype assignment, hence up-regulation if \( ES > 0 \) and down-regulation if \( ES < 0 \). The strength of GSEA and its application to microarray data is that multiple genes belonging to particular gene sets, which are defined based on prior biological knowledge (e.g., genes encoding products in a metabolic pathway, located in the same cytogenetic band, or sharing the same GO category), rather than individual genes are used to assess for co-ordinate gene expression between two conditions of interest, bridging microarray data with biological significance.

References
4. Raychaudhuri S, Stuart JM, Altman RB. Principal components analysis to summarize microarray experiments: application to sporulation time series. Pac Symp Biocomput 2000; 455-466


Legends to Supplementary Figures

Supplementary Figure 1. Microarray analysis documents enrichment of stemness in TICs. Microarray GSEA enrichment plots and LEGs of significantly enriched NSC, ESC and HSC in MCF7 spheres, NSC in IstMes2 spheres and NSC and HSC in LNCaP spheres.

Supplementary Figure 2. qPCR validation of stemness LEGs. MCF7, IstMes2, LNCaP adherent cells and spheres were assessed for stemness LEGs mRNA by qPCR. The data are derived from three independent experiments and are represented as mean values ± S.D. The symbol ‘*’ indicates statistically significant data for the spheres compared to their adherent counterparts.

Supplementary Figure 3. Primary GBM cells were isolated from two patients (B30 and B31). The top left panels show the hematoxyllin/eosin staining of the tumor sections, the middle left and bottom left panels present adherent and sphere cells, respectively, derived from the B30 and B31 patient biopsies. The right panels document the fold-change in the level of indicated genes assessed by qPCR in sphere cells related to adherent cells. The data are derived from three independent experiments and are represented as mean values ± S.D. The symbol ‘**’ indicates statistically different data for the adherent and corresponding sphere cells. The images are representative of at least three independent experiments.
Supplementary Figure 4. Effect of α-TOS and MitoVES on IDO2. MCF7, IstMes2 and LNCaP cell-derived spheres were exposed to 5 μM MitoVES or 50 μM α-TOS for 3 and 6 h and assessed for the level of IDO2 mRNA using qPCR. The data are derived from three independent experiments and are represented as mean values ± S.D.

Supplementary Figure 5. A. The putative binding sites of IDO1 mRNA 3’UTR for individual MiRs. B. The level of expression of stemness MiRs in sphere cells relative to their adherent counterparts. C. The level of expression of the ZEB1 protein relative to their adherent counterparts. The data are derived from three independent experiments and are represented as mean values ± S.D. The symbol ‘*’ indicates statistically different data for the adherent and corresponding sphere cells.

Supplementary Figure 6. Analysis of the promoter region of the IDO1 gene for putative binding sites of transcriptional factors.
Figure S3

Figure S4
Figure S5

A

3' acaauuacgaauuACAUCCuc 5' hsa-miR-155
135:5' accugugcaauuuca8GAC66a 3' ID01

3' agGGGUGUCCGGGGGC66c 5' hsa-miR-760
60:5' uAACAUG68GACAGGCAa 3' ID01

3' guuACAUCCGU--UGC-GGUGu 5' hsa-miR-455-5p
1:5' ---UG9ACCCCAACGAGCACu 3' ID01

3' cuagugaaaaacacuAGuAGG6u 5' hsa-miR-153
76:5' gccacaauuACUAUGCAa 3' ID01

B

C

Figure S6

IDO1

1: IRF1; 2: IPF1, DEAF1; 3: IRF7A, SMAD4; 4: XPF1, ZEB; 5: DEAF1;
6: NERF1a,7: Myc-Max; 8: IRF1; 9: Unknown conserved sequence
Discussion and Conclusion

Cancer is a major cause of death in the western world and is becoming an increasing problem. It is now the number one reason for the demise of human patients, having surpassed the number of deaths linked to cardiovascular diseases (Twombly, 2005), and the trend in this sense appears rather grim (Jemal et al., 2010). Even though treatment and therapeutic approaches to cancer have become better and there has been great improvement in the diagnosis of this group of pathologies, many cancer types are still lethal and there is no substantial treatment in many cases. Further, even if treatment is successful there is a high risk of tumour recurrence. Tumours, having been exposed to the first-line of therapy, carry on resistance when they ‘re-appear’ thus making them even harder to treat.

Recent research over the last decade or so has seen the renaissance of the CSC theory. This theory had been proposed over a century ago, suggesting that cancer might arise from a rare cellular sub-population with stem-like characteristics, although it has not been until quite recently that this notion has resurfaced. Cancer is now regarded as a disease of hierarchical, heterogeneous populations made up of both fast-proliferating committed cells and slow-proliferating cells with features of somatic stem cells, which are capable of self-renewal, asymmetric division and multilineage differentiation (Hanahan and Weinberg, 2011). These cells have been shown to have the ability to self-renew and differentiate, and to have high levels of telomerase and DNA repair activity. They are also able to activate anti-apoptotic pathways and have increased membrane transporter activity (ABC drug transporters), giving these cells increased levels of drug resistance and hence, making them resistant to currently used treatments. These cells also have the ability to migrate and metastasise, giving rise to secondary tumours and last, but certainly not least, they have the ability to evade the immune system, a feature, which makes these cells invisible to the immune system and hence allows them to successfully form secondary tumours.

There are several markers used to characterise CSCs (Table 5). Some of these markers are tissue specific, some are used to define CSCs from several tissues. One of the markers used to characterise various CSCs is CD133. Several studies have reported that CD133+ cancer cells are able to form tumours in vivo in NOD/SCID mice with only $10^3$ or less cells being required, whereas when the cancer cell population comprises both CD133+ and CD133- cancer cells at least $10^7$ cells are needed for tumour formation (Yin
et al., 2007). Also, a study by Liu et al. (2006) showed that CD133+ glioblastoma cells from cancer patients expressed higher levels of anti-apoptotic proteins such as FLIP and lower level of the pro-apoptotic protein Bax (Table 6).

In accordance with these findings, the studies reported in this thesis show that the percentage of cells expressing the anti-apoptotic protein FLIP was increased in CD133\textsuperscript{high} Jurkat cells by about 60% and decreased in CD133\textsuperscript{low} Jurkat cells by about 15%, while this was about 20% for the unseparated population (Publication 1). Since FLIP is an inhibitor of the death receptor-mediated apoptotic pathway, which can be induced by TRAIL, an immunological inducer of apoptosis, the responsiveness of CD133\textsuperscript{high} and CD133\textsuperscript{low} cells to TRAIL-mediated treatment was tested. As was expected, CD133\textsuperscript{high} cells were resistant to TRAIL, while CD133\textsuperscript{low} cells were sensitive. Due to the fact that increased percentage of cells expressing the FLIP protein may be responsible for low responsiveness to TRAIL treatment, the percentage of cells expressing FLIP was decreased to determine whether this would sensitise the otherwise resistant cells to the immunological apoptogen. The percentage of cells expressing FLIP in CD133\textsuperscript{high} Jurkat cells was decreased using FLIP-specific siRNA. These cells were then exposed to TRAIL and the results revealed that decreased percentage of FLIP\textsuperscript{+} cells sensitised these cells to the treatment. Therefore, these results show that CD133\textsuperscript{high} cells, which may represent CSCs as has been shown for various cancer types (Table 5), are resistant to TRAIL due to increased number of cells expressing FLIP, an inhibitor of death receptor-mediated apoptosis, and that decreased expression of FLIP using siRNA sensitises CD133\textsuperscript{high} Jurkat cells to apoptosis induced by the death ligand. The results from this study are also consistent with a recent report by Geserick et al. (2008), which showed that downregulation of FLIP itself in melanoma cells resistant to TRAIL was sufficient to sensitise the cells to this immunological apoptogen (Geserick et al., 2008).

Generally, CSCs may have acquired resistance to killing by death ligands, such as TRAIL, as a result of the interaction between the tumour cells and the immune system, which is a source of death ligands, so that a sub-set of cells with resistance to ligands like TRAIL developed in order to evade tumour immunosurveillance and give rise to tumours (Chan and Housseau, 2008).

CD133 has been in the focus of research for some time due to its association with CSCs. Notwithstanding its importance, the exact role of CD133 in CSCs has not been determined thus far. CD133 was first isolated from haematopoietic stem cells by a monoclonal antibody recognizing a specific epitope designated as AC133. Despite the
fact that CD133 is found in many cell lines and most adult tissues with the exception of mature peripheral blood leukocytes (Shmelkov et al., 2004), the AC133 antigen expression is more restricted to undifferentiated cells, including endothelial progenitor cells (Peichev et al., 2000), hematopoietic stem cells (Yin et al., 1997), fetal brain stem cells (Uchida et al., 2000), embryonic epithelium (Corbeil et al., 2000; Weigmann et al., 1997), prostatic epithelial stem cells (Richardson et al., 2004), myogenic cells (Torrente et al., 2004), and found in certain cancers such as retinoblastoma (Maw et al., 2000; Miraglia et al., 1997), teratocarcinoma (Miraglia et al., 1997) and leukaemias (Bhatia, 2001), as the AC133 antibody recognizes only a particular glycosylated form of CD133 (Yin et al., 1997). Furthermore, the AC133 antibody may selectively bind to a unique alternatively spliced isoform of CD133, and this isoform may be specific to undifferentiated cells as well as many different CSCs, in which CD133 has now been found and is used for their identification and isolation, as was performed in the studies in all publications in this thesis. There is a number of alternatively spliced isoforms of CD133, which have been identified both in mice and humans, and it has been shown that these have tissue-specific distribution (Corbeil et al., 2001; Shmelkov et al., 2004; Yu et al., 2002). However, regardless of the cell type in which CD133 is expressed, it was shown that CD133 is restricted to cellular protrusions (Corbeil et al., 2001).

Various antibodies have been developed that target different epitopes and isoforms of CD133 (Chen et al., 2010; Swaminathan et al., 2010; Wang et al., 2010), however most of these antibodies are still formed to recognise a glycosylation-dependent CD133. Recently, Swaminathan and colleagues developed an antibody, which recognises the unmodified CD133 extracellular domain and not its glycosylated epitope (Swaminathan et al., 2010). Nevertheless, for CSC identification and isolation, also used in this thesis, the AC133 antibody has been used most frequently.

In epithelial and other cell types, proteins from the CD133 family are associated with cholesterol-rich lipid rafts, suggesting a function in the organisation of plasma membrane topology (Weigmann et al., 1997; Röper et al., 2000; Corbeil et al., 2001; Mizrak et al., 2008; Corbeil et al., 2010). CD133 has also been proposed to be involved in several signalling pathways (Tang et al., 2007; Mizrak et al., 2008), such as the Notch pathway (Fan et al., 2010; Sullivan et al., 2010) and the related sonic Hedgehog/Gli pathway (Clement et al., 2007), both of which play a crucial role in normal stem cell as well as CSC proliferation and self-renewal. These observations have been recently supported by a study that showed that blocking the Notch pathway
resulted in the downregulation of CD133, which precluded these cells from forming spheres and from initiating tumours in immunocompromised animals (Fan et al., 2010; Sullivan et al., 2010). In the present study CD133\(^+\) mesothelioma cells, grown as spheres, had an increased level of Notch as well as an increased level of CD47 (Neuzil et al., unpublished), which is a marker of resistance of cancer cells to the phagocytosis by macrophages, giving extra protection of CSCs from the immune system (Chan et al., 2009; Jaiswal et al., 2009). This suggests another link between CD133\(^+\) cells and the propensity of CSCs to evade immune surveillance.

CD133 has also been suggested to be involved in adaptive changes in cellular bioenergetic metabolism, providing CSCs with increased survival advantages. This follows from findings that there is a great increase in the proportion of CD133\(^+\) cells in the population based on FACS analysis in the presence of high glucose, whose uptake is facilitated by a CD133-dependent process (Zhu et al., 2001). It has recently also been documented that CD133 expression reveals a state of hypoxic induction in CSCs and may be indicative of a side population of stem cells, which are transient in nature depending on the prevailing microenvironment and conditions these cells are exposed to and influenced by (Matsumuto et al., 2009; Soeda et al., 2009). Furthermore, increased exposure to hypoxia, which stabilises the hypoxia-inducible factor-1\(\alpha\) (HIF-1\(\alpha\)), is linked to an increase in the proportion of CD133\(^+\) cells in the population based on FACS analysis (Griguer et al., 2008; McCord et al., 2009; Soeda et al., 2009), and activation of HIF-1\(\alpha\) in response to hypoxia and increase in the proportion of CD133\(^+\) cells requires activation of the Akt kinase. Due to the fact that Akt is a central regulator of the survival pathways, promoting survival and proliferation while suppressing apoptosis, it is proposed that increase in the amount of CD133\(^+\) cells is linked to increased resistance to apoptosis (Publication 2, Figure 2). Furthermore, HIF-1 is known as a regulator of a variety of mitochondrial bioenergetic pathways as well as being a sensor of oxygen (Ralph et al., 2010b; Semenza, 2010), such that the increased activity of HIF-1\(\alpha\) and an increase in the proportion of CD133\(^+\) cells in the population based on FACS analysis connects this resistant phenotype with mitochondrial bioenergetics. CD133 has also been associated with resistance of CSCs to apoptosis (Frank et al., 2005; Bao et al., 2006; Clement et al., 2007; Frank et al., 2010) as already has been discussed in more detail above.

In accord with the reports on the role of CD133 in CSC resistance and promotion of survival, stress in the form of nutrient deprivation, cell ‘crowding’, or
hypoxia was observed to result in apoptosis induction (Neuzil et al., unpublished), and, very interestingly, the surviving population showed an increase in the proportion of CD133$^+$ cells based on FACS analysis. Cancer cells growing in monolayer cultures exposed to the same stresses also resulted in rapid upsurge in the amount of CD133$^+$ cells with ensuing increased resistance to apoptosis. These observations were made in several different cancer cells, including Jurkat T lymphoma, breast cancer and mesothelioma cell lines, suggesting that the varying proportion of CD133$^+$ cells in the populations may signify more general properties of CSCs (Neuzil et al., unpublished). These results indicate that the cells, which do survive, either already displayed the CSC phenotype and were ‘ready’ for this kind of insult or that the pressure of the stressful environment favoured cancer cell populations with the ability to acquire increased cancer stem-like properties in order to survive. Hence, the cells are shaped by the surrounding environment, the prevailing conditions such as oxidative or hypoxic stress and by factors such as the immune system, which has been recently shown to play a very important role in the sculpting of cancer cells that have stem cell features, epitomised by elevated CD133 expression. These cells are also more resistant to killing by cells of the immune system. Further, they acquire resistance to anti-cancer therapy, being capable of formation of second-line tumours. Another possibility stems from the studies of Griguer et al., who demonstrated that CD133 expression was upregulated in response to stress by hypoxia or by inhibition of mitochondrial respiration using rotenone (Griguer et al., 2008).

Based on the various studies and results in Publication 2, a hypothesis is proposed, according to which the surface marker CD133 selects for cancer cells to survive immunosurveillance as well as stress-induced apoptotic challenges (Publication 2, Figure 5). Firstly, cells undergoing transformation are exposed to immunosurveillance, and the cells that emerge from the process of immunoediting are resistant to killing by immunological apoptotic inducers as well as by established anti-cancer agents. These cells with stem cell-like features are CSCs, also showing increased proportion of CD133$^+$ cells. Secondly, cancer cells (including cancer cell lines) are hierarchical: they comprise a sub-population of cells that, upon being challenged with inducers of apoptosis (e.g. oxidative stress, hypoxia, anti-cancer agents) will rapidly upsurge the amount of CD133$^+$ cells and, associated with this, upregulate anti-apoptotic genes and downregulate pro-apoptotic genes, resulting in their survival. These cancer
cells have now acquired stem cell properties, such that they are able to form tumours and that they are more resistant to therapeutic interventions.

CD133 has been used most frequently for the identification of CSCs. However, other markers, both extrinsic (eg. CD44, CD24, CD47) and intrinsic (eg. Oct-4, ALDH1), have been utilised for the identification of CSCs from various cancer types (Table 5). CSCs of solid tumours are presently being examined in vitro by maintaining cancer cells in spheres, which are clusters of cells enriched in CSCs. The special medium that the spheres are grown in maintains their stem-like properties. This model was established for breast and prostate cancer as well as mesotheliomas and glioblastomas (Publication 2; Publication 3; Publication 4; Neuzil et al., unpublished). The breast cancer spheres (mammospheres) were first characterised using several different markers of stemness including CD133/1, CD133/2, CD44, CD24, Jagged-1 and Notch-1 (Publication 2, Figure 3; Publication 3, Figure 3). All these markers except for CD24 were increased in mammospheres in comparison to the adherent breast cancer cell line (MCF7). In this model, CD24 was down-regulated; however its status in the mammosphere model can vary (Neuzil et al., unpublished). Both epitopes of CD133 were up-regulated in mammospheres with CD133/2 being also slightly up-regulated in adherent cells. CD133/1 and CD133/2 recognise different epitopes of the CD133 protein, with CD133/1 being the early lineage marker, as the antibody CD133/1 is an AC133 clone; as discussed above, the AC133 antigen is more restricted to undifferentiated cells (Yin et al., 1997). This is the reason why CD133/1 is highly expressed only in mammospheres. Next, microarray analysis was used to characterise the stemness of breast and prostate cancer cells, as well as mesothelial cells grown as spheres (mammospheres, prostate spheres and mesospheres, respectively) (Publication 2, Figure 4; Publication 3, Figure 4; Publication 4, Figure 1). Principal components analysis (PCA) revealed two separate populations of the adherent and sphere cells for the three cell lines. Gene set enrichment analysis (GSEA) based on the enrichment of the stemness gene sets for neuronal stem cells (NSC), embryonal stem cells (ESC) and haematopoietic stem cells (HSC) confirmed an overall increase in the ‘stemness signature’ of these cultures, demonstrating an enrichment in markers of several types of stem cells, including the HSC, ESC and NSC gene sets (Ramalho-Santos et al., 2002; Ivanova et al., 2002; Fortunel et al., 2003). This approach is also very useful in characterisation of global features that are shared by the different types of CSCs, including the pathways that become activated. An example of such pathway found to be
the most activated of all pathways and common to the three CSC types (breast, prostate and mesothelial CSCs) was the tryptophan metabolism pathway, suggesting a mechanism by which CSCs may survive for prolonged periods of time in the niche. Other pathways that were also shared in the three CSC models were glycolysis and oxidative phosphorylation (Publication 2, Figure 4; Publication 3, Figure 4; Publication 4, Figure 1; http://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE41980). Hence, not only upregulation of different markers but also increased activation of various pathways may give these cells stem-like properties and endow them with increased resistance to most currently used anti-cancer therapies, including radiation therapy as well as chemotherapy.

The search for effective anti-cancer agents that would eradicate not only fast-proliferating cancer cells but also slow-growing cancer cells with stem-like properties (CSCs) is of paramount importance. To date, not many successful agents have been found, and several potentially promising compounds have been described (Publication 3, Table 1). One of the first compounds that was found to successfully suppress the proliferation and induction of apoptosis in leukaemia stem cells, firstly, was parthenolide (Guzman et al., 2005a,b, 2007), recently it has also been shown to efficiently eradicate breast and prostate CSCs (Liu et al., 2008; Zhou et al., 2008; Kawasaki et al., 2009). Several other agents have been trialled such as salinomycin, which was able to induce apoptosis in resistant cells (Fuchs et al., 2009). However, this compound is considerably toxic (Li et al., 2010), so its use is questionable. Metformin has also been shown to target breast CSCs and in combination with doxorubicin prevent tumour growth as well as remission (Hirsch et al., 2009). Some of these agents act by inducing ROS. This observation is compatible with results obtained with anti-cancer agents that were examined in this thesis. These compounds, belonging to the group of vitamin E analogues, are epitomised by the redox-silent α-TOS (Figure 27). α-TOS acts by targeting the mitochondrial CII, causing generation of high levels of ROS, and apoptosis induction by destabilising the MOM (Dong et al., 2008, 2009), as has been described in the literature review in chapter two. Recently α-TOS was modified by tagging it with the TPP+ group, which targets this compound directly to the mitochondrial CII due to docking of TPP+ at the matrix face of the MIM, generating a compound referred to as MitoVES (Figure 29). Due to the fact that MitoVES accumulates in mitochondria, it was 1-2 log more efficient in killing cancer cells than the untargeted counterpart (α-TOS). This was paralleled by an effect on experimental
cancer, including colon cancer and HER2-high breast cancer (Dong et al., 2011a). In light of these results and the fact that some potential anti-CSC agents act by inducing ROS, the effect of both α-TOS and MitoVES was tested on CSC models, represented by the spheres cultures. MitoVES was very efficient in inducing apoptosis in mammospheres, in comparison to the untargeted α-TOS, which had hardly any effect on these spheres (Publication 2, Figure 3; Publication 3, Figure 3). In fact, MitoVES was even more efficient in killing mammospheres than parthenolide (PTL) (Publication 2, Figure 3; Publication 3, Figure 3). PTL did not have much effect on adherent MCF7 cells with about 25% apoptosis after 24 h incubation. In comparison to this α-TOS treatment resulted in about 50% apoptosis after 24 h and MitoVES in about 80% apoptosis. In mammospheres the situation was slightly different with PTL being about 2-fold more effective than α-TOS. MitoVES was considerably more efficient in inducing apoptosis in comparison to the other two agents. MitoVES treatment resulted in increased level of apoptosis already after 2 h incubation with about 85% of mammospheres being in apoptosis after 8 h incubation. The level of apoptosis remained similar for the next two time points, 12 and 24 h. On the other hand, PTL treatment resulted in about 55% apoptosis after 24 h incubation, indicating that MitoVES is truly more efficient in inducing apoptosis in mammospheres. Hence, these results suggest that MitoVES has a considerable potential in CSC treatment. Furthermore, the effect of α-TOS and MitoVES on prostate spheres and mesospheres was also tested with MitoVES being very effective in inducing apoptosis in both these sphere types, while the effect of α-TOS was also minimal (Neuzil et al., unpublished data). These results indicate that MitoVES shows a substantial promise to be used as a compound that will efficiently eradicate not only the bulk of the tumour cells but, more importantly, also the highly recalcitrant and resistant CSCs, and in this way minimise the probability of tumour remission.

As mentioned above, the microarray analysis of the three CSC models (mammospheres, prostate spheres and mesospheres) revealed that the Trp metabolism is the most activated pathway in all the three models, represented by the increased expression of the rate-limiting enzyme IDO (Publication 4, Figure 1 and Figure 2; http://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE41980). This is a very intriguing result as depletion of Trp is one way cancer cells could protect themselves from the immune surveillance, giving cancer cells both passive and active defense mechanisms (Munn and Melor, 2007; Löb et al., 2009). The increase of Trp metabolism
causes a depletion of this essential amino acid from the tumour environment, resulting in suppression of cytotoxic T cells and maturation of naïve T cells into T_{reg} cells (Katz et al., 2008; Löb et al., 2009), resulting in protection of the tumour. Therefore, suppressing the enhanced rate of Trp metabolism (for example by inhibiting IDO) using small molecules ought to have an anti-tumour effect, since it would make cancer cells more susceptible to killing by the cells of the immune system (Kumar et al., 2008; Muller et al., 2010; Pilotte et al., 2012). Indeed, inhibitors of IDO, such as brassinin or 1MT, are being considered as anti-cancer drugs (Gaspari et al., 2006; Hou et al., 2007).

TDO is also the rate-limiting enzyme in the Trp metabolism pathway. Until recently, the function of TDO was considered to degrade excess Trp in the liver. However, several studies now have demonstrated that TDO also plays a role in mediating anti-tumour immune responses (Opitz et al., 2011), and that it is constitutively expressed in several cancers including glioma, B-cell lymphoma, Ewing sarcoma, melanoma, hepatocarcinoma, and bladder, cervical, colorectal, lung and ovarian carcinomas (Opitz et al., 2011; Pilotte et al., 2012). In fact, a promising TDO inhibitor with high selectivity, and good oral bioavailability has been developed (Dolusic et al., 2011).

Microarray analysis revealed that TDO2 is a leading-edge gene in prostate spheres (Publication 4, Figure 1; http://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE41980). However, based on various studies, which demonstrate the key role of IDO in cancer immunity, and expression of IDO and TDO on the mRNA level (data not shown for TDO; Publication 4, Figure 2 for IDO), IDO was selected for further study and analysis in this thesis.

Using the three CSC models as well as two primary glioblastoma sphere models, an increase in the expression of IDO in the spheres in comparison to their adherent counterpart was confirmed both on the mRNA and protein levels (Publication 4, Figure 2). This was consistent with the results from the microarray analysis and indicates that these cells are able to ‘hide’ themselves from the immune system. Since mitocans (α-TOS and MitoVES) induce apoptosis in cancer cells as well as CSCs, the effect of these compounds on the expression of IDO was examined. The results clearly showed that both α-TOS and MitoVES were able to significantly suppress expression of IDO in all types of the sphere cells (Publication 4, Figure 3). Furthermore, the regulation of IDO expression in CSCs and its suppression by mitocans was found to be both transcriptional as was analysed by the ChIP assay and post-transcriptional as was analysed by microRNA assay (Publication 4, Figure 5), depending on the type of
tumours. Due to the fact that Trp is an essential amino acid, its increased metabolism in CSCs ought to be compensated for by its enhanced uptake. This was indeed the case as both the LAT1 and CD98 genes, whose products constitute the Trp transporter, were significantly increased both on the mRNA and protein levels in all five sphere models, which was correlated by an increased level of Trp uptake in all sphere models tested (Publication 4, Figure 6). The increase of LAT1 and CD98 was firstly shown in the microarray data (http://www.ncbi.nlm.nih.gov/geo/ with the accession number GSE41980). Finally, an intriguing result was achieved using mice with serially transplanted tumours, which demonstrated an increase in the expression of IDO with each generation of tumours, such that in generation four it was almost 10-fold higher than in generation one. Also the lag time from engraftment of the cells to tumour appearance (detected by ultrasound imaging) shortened considerably with each generation, suggesting that the tumour cells were gradually acquiring a more aggressive phenotype. Altogether, these results indicate that model CSCs derived from several unrelated cancer cell lines as well as primary glioblastoma biopsies increase the level of IDO, most likely to evade the immune system, re-program the T cells in their favour, which may result in long-term escape of CSCs from immune surveillance (Fallarino et al., 2002), and promote their survival to form second-line tumours. However, this can be reversed by mitocans, which lower the level of IDO and hence would consequently enable the immune system to ‘see’ the tumour cells, removing their defense to make them more vulnerable to elimination by the immune cells.

In conclusion, the data presented here clearly document that breast, prostate and mesothelial CSCs as well as primary glioblastoma CSCs grown in the form of spheres have increased stemness as demonstrated by the results from the microarray analysis as well as by using several different markers such as CD133. These cells exerted increased resistance to apoptosis due increased levels of FLIP and upregulated IDO, endowing them with increased propensity to evade the immune system. Mitocans were shown to overcome this ‘evasive’ nature of CSCs by lowering the increased levels of IDO; these agents are also able to trigger efficient apoptosis in these cells. It is therefore very tempting to speculate that a highly efficient way to eradicate tumour cells, including both fast-proliferating cancer cells and the resistant CSCs, may be by using anti-cancer agents like MitoVES that would kill the bulk of the tumour cells, as well as inhibit IDO, which would allow for the cells of the immune system to attack the remaining tumour cells. Combining MitoVES with other IDO inhibitors might be even more effective.
Despite the notion that a lot of work is yet to be undertaken, it is plausible to propose that MitoVES alone and, in particular in combination with other agents, may present an efficient anti-tumour strategy that would deliver what is needed: act via an invariant target to kill cancer cells and CSCs, minimising the probability of the recurrence of tumours.
8 Future Directions

Cancer is now the number one reason for the demise of human patients in the western world and it is quickly becoming an increasing problem world-wide. Current treatment options are not always successful and in many cases even if first-line therapy is successful the tumour can relapse. CSCs have been suggested as the reason behind more resistant tumours and tumour recurrence. The data presented in this work point to the mitocan MitoVES as being a promising anti-cancer agent, which is able to induce apoptosis not only in cancer cells but also in CSCs. MitoVES is also able to suppress the expression of IDO in these cells and in that way make them ‘visible’ for elimination by the immune system. Thus far, MitoVES as well as α-TOS have been examined for their effects, and found that results with MitoVES are more promising. This follows largely from *in vitro* studies of several different cancer cell lines as well as cancer cells from primary glioblastoma biopsies.

The molecular mechanism of the induction of apoptosis in response to MitoVES has been studied rather extensively (Dong *et al.*, 2011a,b). The effect of MitoVES on the Trp metabolism pathway and T cells, however, needs to be further studied, so that it can be better understood how MitoVES aids the immune system and what impact it could have on the potential translational aspects. In particular, the question remains whether MitoVES will cause a decrease of T<sub>reg</sub> cells and increase of cytotoxic T cells. The effect of MitoVES *in vivo* has to be thoroughly examined. The experiments should include athymic, nude mice for xenografting of different cancer types such as mesothelioma or glioblastoma, which have impaired immunity, in order to see the effect of MitoVES only on the tumour. Next, transgenic mice for breast and prostate cancers or C57BL mice with syngeneic tumours should be used as these animals are immunocompetent, hence the effect of MitoVES on the immune system *in vivo* can also be studied. In the latter case MitoVES treatment can be combined with other agents such as IDO inhibitors, which could have a greater impact and could also give more information about the interaction with the immune system and, as well, whether under this setting cells of the immune system contribute to tumour demise. Growth of the tumour in control or treated mice should be monitored and quantified using the ultrasound imaging system, which is very precise and allows for unambiguous and non-invasive quantification of whole tumours, more or less regardless of their location. The expression of different markers such as CD44 or proteins of interest such as IDO in the
tumour tissue as well as the carcinoma microenvironment should be assessed using qPCR and fluorescent/confocal microscopy of tumour sections. These are only several of the possible avenues sprouting from the results of this project.

In conclusion, given the results achieved to date, additional data (yielded for example by the experiments indicated above) should result in the application of these findings to a translational shift of the project, such that pre-clinical and, potentially, clinical tests are run. It is hoped that this may provide a new paradigm of efficient cancer treatment in the near future as these novel drugs enter the clinic.
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emedicinehealth


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Appendices

Appendix 1: The TNM classification system for staging different types of cancer based on certain standard criteria (American Joint Committee on Cancer, 2010).

The TNM Staging System is based on the extent of the tumour (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

The T category describes the original (primary) tumour.

<table>
<thead>
<tr>
<th>TX</th>
<th>Primary tumour cannot be evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ (early cancer that has not spread to neighbouring tissue)</td>
</tr>
<tr>
<td>T1–T4</td>
<td>Size and/or extent of the primary tumour</td>
</tr>
</tbody>
</table>

The N category describes whether or not the cancer has reached nearby lymph nodes.

<table>
<thead>
<tr>
<th>NX</th>
<th>Regional lymph nodes cannot be evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>No regional lymph node involvement (no cancer found in the lymph nodes)</td>
</tr>
<tr>
<td>N1-N3</td>
<td>Involvement of regional lymph nodes (number and/or extent of spread)</td>
</tr>
</tbody>
</table>

The M category tells whether there are distant metastases (spread of cancer to other parts of the body).

<table>
<thead>
<tr>
<th>MO</th>
<th>No distant metastasis (cancer has not spread to other parts of the body)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Distant metastasis (cancer has spread to distant parts of the body)</td>
</tr>
</tbody>
</table>

Each cancer type has its own classification system, so letters and numbers do not always mean the same thing for every kind of cancer. Once the T, N, and M are determined, they are combined, and an overall ‘Stage’ of I, II, III, IV is assigned. Sometimes these stages are subdivided as well, using letters such as IIIA and IIIB.
Appendix 2: Methods and Materials for Chapter 4 and 5

**Cell Culture and Treatment**

MCF7 cells were grown in the DMEM medium supplemented with 10% FBS, 1% antimycotics and antibiotics, and maintained at 37°C and 5% CO₂. MCF7 spheres were cultured in the DMEM-F12 medium, with added Neurocult Neural Stem Cell Proliferation Supplement, 20 ng/ml rhEGF, 10 ng/ml rhFGF and 1% antibiotics, and maintained at 37°C and 5% CO₂.

For apoptosis detection, MCF7 adherent cells and spheres were treated with 50 μM α-tocopheryl succinate (α-TOS), 5 μM mitochondrially targeted vitamin E succinate (MitoVES) and 10 μM parthenolide (PTL) for 2, 4, 6, 8, 12 and 24 h.

**Flow Cytometric Analysis of Protein Expression**

The level of expression of the CD133/1, CD133/2, CD44, CD24, Jagged-1 and Notch-1 proteins was assessed by flow cytometry. MCF7 adherent cells and spheres were harvested, washed with PBS and incubated for 30 min at room temperature (RT) with neutral-buffered formalin (NBF; 3.7% paraformaldehyde in PBS, pH 7.4). They were then incubated with anti-CD133/2 IgG (clone 293C3; Miltenyi Biotec), anti-CD133/1 IgG (clone AC133; Miltenyi Biotec), anti-CD44 IgG (clone 2C5; R&D Systems), anti-CD24 (clone SN3 A5-2H10; eBioscience), anti-Jagged-1 (clone C-20; Santa Cruz) and anti-Notch-1 (clone C-20; Santa Cruz) for 60 min on ice followed by incubation with secondary FITC- or PE-conjugated IgG (Santa Cruz) for 60 min on ice. The cells were then scored by flow cytometry (FACSCalibur, Becton Dickinson) for populations with high and low fluorescence.

**Apoptosis Detection**

Apoptosis was estimated routinely with flow cytometry using the annexin V-FITC (PharMingen) binding method based on phosphatidylserine (PS) externalisation at the relatively early phases of apoptosis. Briefly, MCF7 adherent cells and spheres were harvested, washed with PBS and incubated with annexin V mixture (PBS, annexin-binding buffer (10X), annexin V-FITC conjugate) for 15 – 30 min on ice in the dark. The cells were then assessed for green fluorescence by flow cytometry.
Gene Microarray Analysis
This method is described in the Supplementary text of Chapter 6 – Publication 4.