The development of intratumoral heterogeneity in ovarian tumors: role of cancer stem cells in disease progression

Lunsford, Elaine Patricia
THE DEVELOPMENT OF INTRATUMORAL HETEROGENEITY IN OVARIAN TUMORS: ROLE OF CANCER STEM CELLS IN DISEASE PROGRESSION

by

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B.S., Rensselaer Polytechnic Institute, 2008

Submitted in partial fulfillment of the requirements for the degree of

Master of Science

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ACKNOWLEDGEMENTS

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In addition, Drs. Theresa Davies and Gwynneth Offner, for granting me a “second chance” to prove my readiness for medical school. I appreciate deeply the help I’ve received from the MAMS program in reaching for my dreams.
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ELAINE P. LUNSFORD

ABSTRACT

Like with many cancers, a single ovarian tumor can display remarkable diversity in genetics, epigenetics, expression profiles, microenvironment and cell differentiation and plasticity. This so-called intratumoral heterogeneity (ITH) is thought to greatly increase mortality by enabling tumors to adapt quickly to therapy, metastasize, and recur, thus the study of ITH holds great clinical significance. Clonal evolution and cancer stem cell (CSC) theory are two models for the initiation and propagation of a tumor, which offer differing views on the way that ITH is developed and maintained. In the clonal evolution model, cancer arises from a single cell and, through genetic instability, proliferates into a diverse population of daughter cells, which develop additional mutations and undergo Darwinian selection under the influence of the tumor microenvironment. Each cell of the clonal evolution model may be capable of initiating a tumor independently. In CSC theory, cancer arises from the transformation of a stem cell that has the capacity to self-renew and differentiate into a diverse population of daughter cells. Each cell is NOT capable of tumorigenesis as most are terminally differentiated and do not harbor self-renewing capabilities. According to CSC
theory, small, rare subpopulations of CSCs persist throughout chemotherapy and are responsible for repopulating the heterogeneous tumor post-treatment. The hypothesis that CSCs may play a role in ovarian cancer progression is the subject of this thesis. Many studies have detected the presence of stem cell markers and dysregulated stem cell signaling pathways in ovarian cancer, but doubts remain as to the existence of ovarian CSCs; critics have pointed out inherent flaws in experimental designs meant to identify and characterize CSCs. For example, the presence of cancer cells which express the stem cell marker CD133 has been correlated to both positive and negative impacts on prognosis. Further challenging the study of ovarian CSCs is the lack of consensus on the true cell of origin for ovarian cancer – whether it be from the fallopian tube epithelium or ovarian surface epithelium, or elsewhere in the peritoneal cavity – this will have important implications for the identification and characterization of tumorigenic ovarian CSCs. Advocates of clonal evolution theory have put forth incredible effort to reveal the extent of inter and intra-tumoral heterogeneity in ovarian cancer, and from these data there has arisen a general consensus that cancer cell populations do evolve in a step-wise fashion, accumulating additional mutations over time. The involvement of cancer stem cells in this progression and how exactly they fit in (as a cell of origin or arising from genetic mutations), as well as their significance for different cancer types, is a question worth answering. Despite the challenges facing the study of ovarian CSCs, the clinical impact of cells with stem-like properties has been repeatedly demonstrated,
especially with regard to metastatic processes and chemoresistance. Moreover, new drugs which target stem cell pathways have proven effective in the treatment of ovarian cancer. The existence of a rare subset of cells that have enhanced tumor-initiating properties is apparent in ovarian cancer, and more work is needed to characterize the unique identifiers and behavior of these cells in vivo. Future experiments involving lineage tracing promise to deepen our understanding of the nature of ovarian CSCs and address whether normal stem cells might serve as the cell of origin.
# TABLE OF CONTENTS

TITLE PAGE ........................................................................................................................................... i
COPYRIGHT PAGE ................................................................................................................................. ii
READER’S APPROVAL PAGE ................................................................................................................... iii
ACKNOWLEDGEMENTS ............................................................................................................................... iv
ABSTRACT ................................................................................................................................................... v

## TABLE OF CONTENTS

- The Importance of Intratumoral Heterogeneity................................................................. 1
- Major Concepts and Discoveries in the Field of Intratumoral Heterogeneity..... 4
- Models for Intratumoral Heterogeneity: Clonal Evolution Theory ....................... 11
- Models for Intratumoral Heterogeneity: Cancer Stem Cell Theory ..................... 15
- Specific Aims ................................................................................................................................. 19

## INTRODUCTION

- INTRATUMORAL HETEROGENEITY IN OVARIAN CANCER................................. 20
  - Ovarian Cancer Subtypes and Pathogenesis............................................................... 20
  - Genetic, Epigenetic and Expression Profiles............................................................. 29
  - Microenvironment........................................................................................................ 39
  - Differentiation and Cell Plasticity............................................................................. 44
THE ROLE OF CANCER STEM CELLS IN HETEROGENEOUS OVARIAN TUMORS

A Review of Stem Cell Biology ................................................................. 46
Stem Cells and Cancer .......................................................................... 55
Evidence for Ovarian Cancer Stem Cells .............................................. 61
Validity of the cancer stem cell model in predicting intratumoral heterogeneity ............................................................................. 69

CONCLUSIONS AND FUTURE DIRECTIONS ........................................... 75

APPENDIX I ................................................................................................ 78
APPENDIX II ............................................................................................ 80
REFERENCES ............................................................................................ 81
CURRICULUM VITAE ............................................................................... 98
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Timeline: Highlights in the Study of Intratumoral Heterogeneity</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Ovarian Cancer Subtypes</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Recurrent Genetic Mutations Found in HGSC</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Regulators of Embryonic Stem Cell Differentiation</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>Qualities of a Cancer Stem Cell</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>Ovarian Cancer Stem Cell Markers</td>
<td>64</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Clonal Evolution Model</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Phylogenetic Tree of Acute Myeloid Leukemia</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>Cell Division and Partitioning of DNA Strands</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Cancer Stem Cell vs. Clonal Evolution Model</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Ovarian Cancer Subtypes</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Hypothesized Progression from Site of Origin in Serous Ovarian Carcinoma</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Pathogenesis of Ovarian Cancer Subtypes from Hypothesized Sites of Origin</td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>Ras-Raf and PI3K-AKT Signaling Pathways</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>Wnt Signaling Pathway</td>
<td>36</td>
</tr>
<tr>
<td>10</td>
<td>The SWF/SNF complex including BAF250A</td>
<td>37</td>
</tr>
<tr>
<td>11</td>
<td>The Cells of the Heterogeneous Tumor Microenvironment</td>
<td>40</td>
</tr>
<tr>
<td>12</td>
<td>Interaction pathways in the ovarian cancer</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>microenvironment</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Key steps in tumor metastasis involve EMT and MET programs</td>
<td>45</td>
</tr>
<tr>
<td>14</td>
<td>Regulatory Pathways in Embryonic Stem Cells</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>Hematopoeitic Stem Cells</td>
<td>48</td>
</tr>
<tr>
<td>16</td>
<td>The Notch Pathway</td>
<td>51</td>
</tr>
<tr>
<td>17</td>
<td>Wnt Signaling Pathway</td>
<td>52</td>
</tr>
<tr>
<td>18</td>
<td>The Hedgehog Pathway</td>
<td>53</td>
</tr>
<tr>
<td>19</td>
<td>TGF-beta Superfamily Signaling Pathways</td>
<td>54</td>
</tr>
<tr>
<td>20</td>
<td>Metastatic Teratocarcinoma</td>
<td>56</td>
</tr>
<tr>
<td>21</td>
<td>Normal Stem Cell Vs. Cancer Stem Cell</td>
<td>58</td>
</tr>
<tr>
<td>22</td>
<td>RT-PCR reveals connection between CD24 and stem cell function</td>
<td>67</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
</tr>
<tr>
<td>BMPs</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>breast cancer type 1/2 susceptibility gene</td>
</tr>
<tr>
<td>CAF</td>
<td>cancer associated fibroblast</td>
</tr>
<tr>
<td>CAF</td>
<td>cancer associated fibroblasts</td>
</tr>
<tr>
<td>CCC</td>
<td>Clear Cell Carcinoma</td>
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<tr>
<td>CGL</td>
<td>Chronic Granulocytic Leukemia</td>
</tr>
<tr>
<td>CK1</td>
<td>casein kinase 1</td>
</tr>
<tr>
<td>CoR</td>
<td>corepressor</td>
</tr>
<tr>
<td>CSC</td>
<td>cancer stem cell</td>
</tr>
<tr>
<td>EC</td>
<td>Endometriod Carcinoma</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>EZH2</td>
<td>enhancer of zeste homolog 2’</td>
</tr>
<tr>
<td>GSIs</td>
<td>gamma-secretase inhibitors</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>HGSC</td>
<td>High Grade Serous Carcinoma</td>
</tr>
<tr>
<td>HSCs</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>ICN</td>
<td>intracellular fragment of Notch</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITH</td>
<td>Intratumoral heterogeneity</td>
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<tr>
<td>LGSC</td>
<td>Low Grade Serous Carcinoma</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>Mucinous Carcinoma</td>
</tr>
<tr>
<td>MET</td>
<td>mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>Pi3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>SBT</td>
<td>serous borderline tumor</td>
</tr>
<tr>
<td>SCNAs</td>
<td>somatic copy number alterations</td>
</tr>
<tr>
<td>SMO</td>
<td>receptor Smoothened</td>
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<tr>
<td>STIC</td>
<td>serous tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TAM</td>
<td>tumor associated macrophage</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor-associated macrophages</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIC</td>
<td>tubal intraepithelial carcinoma</td>
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INTRODUCTION

In the first part of this thesis, intratumoral heterogeneity (ITH) in ovarian cancer will be explored, including contributions of genetic mutations, epigenetic alterations, microenvironment and cell plasticity, and how each of these aspects are critical for disease progression. Next, the extent to which ITH in ovarian cancer can be explained by certain models of tumor progression, namely clonal evolution and cancer stem cell theory, will be assessed using data from the literature. In the following introduction, the clinical relevance and history of the field of ITH will be detailed, and background on the two models of tumor progression, clonal evolution and cancer stem cell theory, will be given.

**The Importance of Intratumoral Heterogeneity**

Ovarian cancer remains a highly lethal disease, with the 5-year survival rate for all types being only 44%, and even less for invasive epithelial types diagnosed at Stage III or IV (Cancer.org (n.d.)). Unfortunately, because women remain largely asymptomatic throughout the early stages of disease, 75% of ovarian cancer is diagnosed at stage III and IV (Marcus, Maxwell, Darcy, Hamilton, & McGuire, 2014). Efforts toward earlier detection have produced mixed results. On one hand, studies point out that screening of asymptomatic women has not proven beneficial and may lead to unnecessary surgery (Reade, Riva, Busse, Goldsmith, & Elit, 2013). Other studies show there is benefit, but limitations in study design render the results inconclusive. Current screening studies using new algorithms
to assess CA-125 levels are underway, with results expected in 2015 (Menon, Griffin, & Gentry-Maharaj, 2014). These new studies show increasing promise toward a recommendation for ovarian cancer screening in the general public which could possibly lead to earlier detection and decreased mortality.

Once diagnosed, patients typically undergo a debulking surgery along with platinum/taxane chemotherapy (Collinson, Seligmann, & Perren, 2012). Despite many patients showing initial sensitivity to treatment, the mostly likely outcome is recurrence and death (Lopez, Banerjee, & Kaye, 2013; Marcus et al., 2014). Thus, as for many cancers, recurrence is a major challenge to the successful treatment of ovarian cancer. Although chemotherapy can successfully eliminate a large bulk of the tumor, certain cells, particularly at advanced stages of the disease, can develop or possess an inherent resistance to treatment (Collinson, Seligmann, & Perren, 2012). The fact that certain cells respond differently to treatment illustrates a widely-known and important aspect of cancer biology – namely that cells within tumors evolve to form a complex mix of subpopulations which differ widely in genetic and phenotypic traits. Some of these traits may confer survival advantages that are responsible for the persistence of cancerous subpopulations during and after treatment. Understanding the nature of these advantages, as well as the mechanisms underlying the development of resistant subpopulations will help in designing more effective treatment strategies.
Intratumoral heterogeneity has also been implicated in metastasis, as well as tumor expansion, relapse, and drug resistance (Brabletz, 2012; Elshamy & Duhé, 2013; Scheel & Weinberg, 2012). Throughout the evolution of a tumor, transformations occur such that cells develop certain “hallmarks of cancer,” including the ability to self-sustain growth factors, evade suppression of growth, escape apoptosis, self-renew, promote and maintain angiogenesis, and finally invade other host tissues and metastasize (Hanahan & Weinberg, 2011). At this stage, the invasive cells are said to be malignant. Many have proposed that these transformed subpopulations evolve in a Darwinian fashion, with cells undergoing a selection process whereby an aggressive, dominant population emerged (Greaves & Maley, 2012; Murugaesu, Chew, & Swanton, 2013).

Current theory suggests, however, that subpopulations which are only partially transformed may work together, in commensal or mutual relationships. This synergy could allow for the hallmarks of cancer to be acquired much earlier, before one subpopulation has become fully transformed, so that metastasis occurs faster than a Darwinian model would predict (Axelrod, Axelrod, & Pienta, 2006).

This theory of cooperation among subpopulations illustrates the importance of understanding intratumoral heterogeneity and the ways in which it can aid in tumor progression. Additional features of intratumoral heterogeneity, such as
contributions from the tumor microenvironment and cell plasticity, play crucial roles in the progression of disease and will be discussed in later sections.

In summary, ITH has important clinical implications for the successful treatment of cancer, for example, while chemotherapy may be effective against a majority of the cancer cells, certain subpopulations can uniquely develop resistance and cause relapse. Further, ITH is theorized to expedite tumor progression toward aggressive metastases, and this process may involve the cooperation of various subpopulations. Understanding the nature of ITH will have major clinical implications, including the potential to design new drugs which may prevent relapse and metastases.

**Major Concepts and Discoveries in the Field of Intratumoral Heterogeneity**

Although the heterogeneous nature of bulk tumors had been recognized for some time, the field of intratumoral heterogeneity gained momentum in the late 1970s and early 1980s, as researchers considered the important implications for treatment and prognosis. Observations of phenotypic diversity were reported in various cancers, and included findings such as differences in proliferation rates (Rabes, Carl, Meister, & Rattenhuber, 1979), variety of cell surface markers (Davis, Zava, Locher, Goldhirsch, & Hartmann, 1984), and protein phosphorylation (Chakrabarty, Jan, Miller, & Brattain, 1985). In each case, the studies emphasized the significance of the findings for treatment response.
The clinical significance of intratumoral heterogeneity is the key behind the continued interest among researchers. As early as 1965, it was asserted that cancer treatment would not be effective without understanding the diversity found within the disease (Foulds, 1965). Toward this end, models of tumorigenesis were proposed, including a Darwinian evolutionary model, whereby genetic instability leads to accumulation of mutations and divergent clonal populations within the same tumor (Nowell, 1976). Others proposed mechanisms in which tumorigenesis bore striking similarity to embryonic development, such that a small population of initiating cells can give rise to a multitude of different cell phenotypes (Nicolson, 1987). Still others emphasized the “societal” aspect of tumors whereby various subpopulations work together to influence tumor growth and behavior (Heppner, 1993).

Adding to the recognized importance of the field was the proposal that intratumoral heterogeneity promoted metastatic processes (Fidler, 1978) as well as drug resistance (Sirachý, 1979). In order to achieve malignancy, it was suggested that a tumor would need to diversify its population to manifest key alterations in cell physiology, termed ‘the hallmarks of cancer’ (Hanahan & Weinberg, 2000). These changes would allow a cancer cell to invade the surrounding stroma, enter the blood stream, and seed new tumor sites elsewhere in the body.
Thus, by the year 2000, the concept that intratumoral heterogeneity is considered a necessary component of cancer progression was emerging, and various models were being developed to explain the mechanisms behind creating this diversity. The key characteristics defining a malignant turning point, the ‘hallmarks of cancer,’ created a framework within which to study intratumoral heterogeneity.

Other major concepts in the field of intratumoral heterogeneity include the contribution of epigenetic alterations – changes that affect gene transcription but do not involve alterations to the genome sequence – as an additional source of intratumoral heterogeneity (Frost & Kerbel, 1983). In parallel, the extracellular environment as well as the inflammatory response were shown to enhance and perpetuate intratumoral heterogeneity, cell proliferation, survival and invasion (Allavena, Sica, Solinas, Porta, & Mantovani, 2008; Gillies, Schornack, Secomb, & Raghunand, 1999; Liotta & Kohn, 2001; Talmadge, 2011).

In the last 10 years, the debate over which model of tumorigenesis best describes the intratumoral heterogeneity found in various cancers has become a hot topic in cancer research. The idea of a ‘cancer stem cell’ has gained popularity and stands to challenge the existing theory of clonal evolution, as well as provide compelling explanations as to the mechanisms behind malignancy and drug resistance (Maugeri-Saccà, Vigneri, & De Maria, 2011; O'Brien, Kreso, & Jamieson, 2010; Soltysova, Altanerova, & Altaner, 2005; Tu, Lin, & Logothetis,
Adding complexity to the cancer stem cell theory has been the most recent evidence for the epithelial-mesenchymal transition (EMT) as a necessary step for metastasis, and the plasticity of cellular differentiation which challenges the traditional hierarchy of ‘one-way’ differentiation originally proposed by the stem cell model (Kreso & Dick, 2014; Marjanovic, Weinberg, & Chaffer, 2013; Polyak & Weinberg, 2009). Cells which undergo an EMT bare striking resemblance to cancer stem cells with regard to cell surface markers and active signaling pathways which control cell behavior, and thus the theory of cancer stem cells has merged to some degree with the EMT, at least with regards to metastatic progression.

The prevailing theories of tumorigenesis, clonal evolution and cancer stem cell theory, will be outlined in the following section. A timeline of the major events and influential papers in the study of intratumoral heterogeneity is given in Table 1.
Table 1. Timeline: Highlights in the Study of Intratumoral Heterogeneity

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
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<tbody>
<tr>
<td>1965</td>
<td>Multiple Epilogeal Factors in Neoplastic Development (Foulds, 1965). “Effective management of neoplastic disease is likely to depend on better knowledge than is yet available of the successive stages of neoplastic development and of the diverse factors operating at each of them.”</td>
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<tr>
<td>1978</td>
<td>Tumor Heterogeneity and the Biology of Cancer Invasion (Fidler, 1978). “The metastatic behavior of tumor cells depends on the responses of the host, the intrinsic properties of the tumor cells, and the microenvironment of the metastatic lesion.”</td>
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<tr>
<td>1979</td>
<td>An Approach to the Problem of Heterogeneity of Human Tumor-Cell Populations (Siracky, 1979). “The tumour-cell population is heterogeneous for sensitivity to various cytostatic drugs or hormones.”</td>
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<td>1983</td>
<td>On a Possible Epigenetic Mechanism(s) of Tumor Cell Heterogeneity. The Role of DNA Methylation. (Frost &amp; Kerbel, 1983). “We herein offer an alternative, but not mutually exclusive, explanation [for tumor heterogeneity] based not on structural gene changes but rather on what has been called ‘epigenetic’ changes.”</td>
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<td>1987</td>
<td>Tumor Cell Instability, Diversification, and Progression to the Metastatic Phenotype: From Oncogene to Oncofetal Expression. (Nicholson, 1987). “Tumor cell diversification mechanisms may be similar or identical to normal developmentally regulated diversification mechanisms that are used during embryonic and postembryonic cell diversification and development.”</td>
</tr>
<tr>
<td>1993</td>
<td>Cancer Cell Societies and Tumor Progression. (Heppner, 1993). “The central conclusion of this work is that tumor subpopulations do not behave independently of each other but rather form a society of cells in which they influence each other’s growth and treatment response. The maintenance of tumor heterogeneity is a consequence of the cancer cell society…cancer behavior and progression depend on interactions among all members of that society, not just on it’s most deviant variants”</td>
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<td>2002</td>
<td>Stem-cell origin of metastasis and heterogeneity in solid tumors. (Tu, Lin and Logothetis, 2002). “An explanation for the inherently metastatic and heterogeneous nature of cancers may be their derivation from distinct stem cells”</td>
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<tr>
<td>2003</td>
<td>Multiple mutations and cancer. (Loeb LA, Loeb KR and Anderson JP, 2003). “Normal mutation rates are insufficient to account for the multiple mutations found in human cancers…cancers must exhibit a mutator phenotype early during their evolution.”</td>
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<td>Year</td>
<td>Event Description</td>
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<td>2005</td>
<td>Cancer stem cells. (Soltysova A, Altanerova V, Altaner C, 2005). “Growth of metastases in distinct areas of the body and their cellular heterogeneity might be consequence of cancer stem cell differentiation and/or dedifferentiation and asymmetric division of cancer stem cells.” Hypoxia-induced dedifferentiation of tumor cells -- A mechanism behind heterogeneity and aggressiveness of solid tumors. (Axelson et al, 2005). “Hypoxia, shortage of oxygen, greatly influences cellular phenotypes by altering the expression of specific genes, and is an important contributor to intra- and inter-tumor cell diversity...we recently observed that hypoxic neuroblastoma cells and breast cancer cells lose their differentiated gene expression patterns and develop stem cell-like phenotypes.”</td>
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<td>2007</td>
<td>The role of epithelial-mesenchymal transition in cancer pathology. (Guarino M, Rubino B, and Ballabio G, 2007). “The essential features of EMT are the disruption of intercellular contacts and the enhancement of cell motility, thereby leading to the release of cells from the parent epithelial tissue. The resulting mesenchymal-like phenotype is suitable for migration and, thus, for tumor invasion and dissemination, allowing metastatic progression to proceed.”</td>
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<td>2009</td>
<td>Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. (Polyak K and Weinberg RA, 2009). “Similar to embryonic development, both EMTs and METs seem to have crucial role in the tumorigenic process. In particular, EMTs have been found to contribute to invasion, metastatic dissemination and acquisition of therapeutic resistance. METs -- the reversal of EMTs -- seem to occur following dissemination and the subsequent formation of distant metastases.” Breast Tumor Heterogeneity: Cancer Stem Cells or Clonal Evolution? (Campbell LL and Polyak K, 2009). “It seems that breast tumor heterogeneity is likely caused by a version of the clonal evolution model that incorporates some features of the cancer stem cell hypothesis.”</td>
</tr>
<tr>
<td>2010</td>
<td>Genome-wide DNA methylation profiles in precancerous conditions and cancers. (Kanai Y, 2009). “DNA methylation alterations at the precancerous stage may confer vulnerability to further genetic and epigenetic alterations, generate more malignant cancers, and thus determine patient outcome.”</td>
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Table 1 continued. Timeline: Highlights in the Study of Intratumoral Heterogeneity

<table>
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<th>Year</th>
<th>Event</th>
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<tr>
<td>2010</td>
<td>How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine (Gerlinger M and Swanton C, 2010).</td>
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<td>&quot;This review summarises the evidence for the evolution of resistance to cytotoxic and targeted anti-cancer drugs according to Darwinian models and highlights the roles of genomic instability and high intra-tumour genetic heterogeneity as major accelerators of this evolutionary process.&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot;It is becoming clear that cancer cells evolve as a result of their ability to hijack normal self-renewal pathways, a process that can drive malignant transformation. Studying self-renewal in the context of cancer and cancer stem cell maintenance will lead to a better understanding of the mechanisms driving tumor growth.&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot;The realization that microRNAs are intimately linked to cancer pathogenesis has spawned an explosion of research activity in recent years. Their presence is not merely predictive of tumor origin and behavior, they are causally linked to the emergence and development of cancer by acting as oncogenes or tumor suppressors.&quot;</td>
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<tr>
<td></td>
<td>Cancer stem cells and chemosensitivity. (Maugeri-Sacca M, Vigneri P, De Maria R, 2011).  &quot;Cancer stem cells seem to be protected against widely used chemotherapeutic agents by means of different mechanisms, such as marked proficiency in DNA damage repair, high expression of ATP-binding cassette drug transporters, and activation of PI3K/AKT and Wnt pathways. Moreover, microenvironmental stimuli such as those involved in EMT and hypoxia indirectly contribute to chemoresistance by inducing in cancer cells a stem-like phenotype.&quot;</td>
</tr>
<tr>
<td></td>
<td>Immune cell infiltration of primary and metastatic lesions: mechanisms and clinical impact. (Talmadge JE, 2011).  &quot;The infiltration of tumors and their metastases by hematopoietic cells can contribute both positively and negatively to tumor growth, invasion, and patient outcomes. These differing outcomes are associated with both tumor heterogeneity and the diversity of leukocytes infiltrating neoplastic lesions.&quot;</td>
</tr>
<tr>
<td>2012</td>
<td>Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. (Gerlinger M et al, 2012).</td>
</tr>
<tr>
<td></td>
<td>&quot;Intratumor heterogeneity can lead to underestimation of the tumor genomics landscape portrayed from single tumor-biopsy samples and may present major challenges to personalized-medicine and biomarker development. Intratumor heterogeneity, associated with heterogeneous protein function, may foster tumor adaptation and therapeutic failure through Darwinian selection.&quot;</td>
</tr>
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Table 1 continued. Timeline: Highlights in the Study of Intratumoral Heterogeneity

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
</table>
| 2013 | The causes and consequences of genetic heterogeneity in cancer evolution. (Burrell RA, McGranahan N, Bartek J, Swanton C, 2013). “Genomic instability is a prominent source of genetic diversity within tumours, generating a diverse population that can be subjected to selection in a given micro-environmental or therapeutic context. There is a crucial need to understand mechanisms driving genomic instability so that therapeutic approaches to limit cancer diversity, adaptation and drug resistance can be developed.”
| 2013 | Cell plasticity and heterogeneity in cancer. (Marjanovic ND, Weinberg RA, Chaffer CL, 2013). “Evolving evidence supports a new model of tumorigenicity, in which considerable plasticity exists between non-cancer stem cell (non-CSC) and cancer stem cell (CSC) compartments, such that non-CSCs can reacquire a CSC phenotype.”
| 2014 | Tumour Heterogeneity and cancer cell plasticity. (Meacham CE, Morrison SJ, 2013). “We do not yet know what fraction of cancers follows the stem-cell model...In some cancers with pervasive genetic heterogeneity, it may not be possible to rigorously test the cancer stem-cell model...Some cancers may have epigenetic heterogeneity that is not well described by the cancer stem-cell model. Indeed, a general question concerns the extent to which the phenotypic and functional properties of cancer cells undergo reversible changes. New models of cancer heterogeneity and plasticity may emerge.”
| 2014 | Evolution of the Cancer Stem Cell Model. (Kreso A, Dick JE, 2014). “Although often considered as mutually exclusive models to describe tumor heterogeneity, we propose that the genetic and cancer stem cell models of cancer can be harmonized by considering the role of genetic diversity and nongenetic influences in contributing to tumor heterogeneity.”
Models for Intratumoral Heterogeneity: Clonal Evolution Theory

During the time that cancer was being redefined as a complex disease of multiple cell types, differing in surface protein expression, DNA ploidy, and genetic makeup, the question of how this progression occurs became of increasing interest. An early yet persistent theoretical model for the evolution of a tumor (Nowell, 1976) built upon the widely held notion that most neoplasms originated from a single cell or 'stem line', and emphasized the role of genetic instability and selection advantages throughout the evolution of ‘clones’ (daughter cells of the original neoplastic cell), suggesting a Darwinian progression (Figure 1). The effect of local tissue environment and drug treatment on tumor evolution, with particular reference toward fostering selection of subclones which persist in metastatic sites in contrast to the primary tumor, is also emphasized. Evidence for this model is supported by cytogenetic data, however at the time, limitations are noted in as much as genetic mapping was unavailable.
Figure 1. Clonal Evolution Model. Tumors evolve from a single normal cell (indicated by ‘N’) which undergoes a transformation that allows it to escape normal cell growth regulation. It rapidly divides, generating clones with increasing genetic instability and mutations. Here, Chronic Granulocytic Leukemia (CGL) is used as an example. Subpopulations which do not survive are indicated by the shaded circles, while those that acquire an additional mutation which confers a survival advantage are indicated by the numbers T1 through T6. Significant biological events and ploidy number are also indicated throughout the progression. Figure taken from (Nowell, 1976).

Today, clonal evolution theory is generally accepted, and advanced versions of Nowell’s depiction of CGL progression are plotted in so-called phylogenetic trees (Figure 2), which contain step-wise progressive genetic mutations that lead to the eventual accumulation of a wide variety of subpopulations both within a single tumor and between patients with the same disease (Shlush et al., 2012).
Importantly, in the clonal evolution model, every cancer cell is capable of generating a tumor, regardless of its position in the evolutionary timeline.

**Figure 2. Phylogenetic Tree of Acute Myeloid Leukemia.** Cells were sampled from two patients before and after treatment, and genetic sequencing revealed mutation progression. With each split in the tree, a new genetic identifier is found in a population of cells. The distance between each split in the tree indicates “cell depth,” and is related to the number of replications the cells underwent before gaining a unique genetic identity from the root. Large depths indicate frequent replication, while shallow depths indicate rare replication of a subclone. Figure taken from (Shlush et al., 2012).
The Swanton group has published extensively on the importance of genomic instability for generating intratumoral heterogeneity, including the selection of the ‘fittest’ clones within a given microenvironment or therapeutic context (Burrell, McGranahan, Bartek, & Swanton, 2013). This mechanism has essentially been proven as an influential force in tumorigenesis, and all other models have incorporated clonal evolution as part of the process alongside new theories of cancer development. Controversy has ensued, however, about the extent to which clonal evolution theory is responsible for intratumoral heterogeneity.

Models for Intratumoral Heterogeneity: Cancer Stem Cell Theory

A stem cell is an unspecialized progenitor which can give rise to a variety of more specialized cells, and is capable of self-renewal. In adults, stem cells make up rare populations that remain relatively dormant in tissues, waiting for queues from the extracellular environment to divide and differentiate. Around the same time that clonal evolution theory began to unfold, investigators were examining stem cells and how mutations in an immortal cell line may be involved in the development of a tumor.

A model was proposed by John Cairns in 1975 in which “immortal” strands of DNA were passed on from one stem cell to its daughter stem cell, while its other daughter cell received a “mortal” strand and went on to terminally differentiate (Figure 3). In this way, genetic mutations could be immortalized and, as they accumulate throughout a person’s lifetime, dramatically increase the risk of
developing cancer, since several mutations are required for cancer to occur (Cairns, 1975; Knudson, Strong, & Anderson, 1973). One can imagine the accumulation of mutations would be many fold higher if a stem cell were to divide into two daughter stem cells, a process called ‘symmetric division’ for example (Kondo, 1977), creating two immortal DNA strands bearing oncogenic mutations instead of one.

Figure 3. Cell Division and Partitioning of DNA Strands. a) normal semi-conservative cell division where each parental DNA strand is randomly assigned to a daughter cell. A mutation is indicated by the double strike. b) stem-cell division where one parental strand (solid line) is preferentially passed on to stem cell progeny, in effect this “older” strand becomes immortalized. The other “younger” strand (dashed line) is passed on to daughter cells which go on to terminally differentiate. A mutation is indicated by the double strike. Taken from (Cairns, 1975).
Today, stem cell theory has taken hold and is a hot topic in the scientific community, although some would argue, little is understood about stem cell differentiation, including Cairns' model of the immortalized DNA strand (Sell, 2004). In brief, cancer stem cell theory proposes that tumors are initiated when a normal stem cell undergoes a transformation which leads to dysregulation of its regulatory mechanisms. As a result, the stem cell divides abnormally, giving rise to either more stem cells, differentiated daughter cells, or both, and creating a diverse tumor environment of various phenotypes. Alternatively, differentiated cells which have adopted a mutator phenotype can de-differentiate and attain stem-like characteristics, which impart the capacity for self-renewal and thus tumorigenesis at metastatic sites as well (Figure 4A). At the heart of the cancer stem cell model is the hierarchy, whereby the tumorigenic stem cell is at the apex, and is capable of self-renewal and capacity to regenerate all phenotypic diversity found in a tumor (O’Brien et al., 2010).
Figure 4. Cancer Stem Cell vs. Clonal Evolution Model. Two approaches for tumorigenesis. A) Classical stem cell theory whereby normal stem cells capable of self-renewal undergo a transformative genetic mutation, creating a cancer stem cell, which can differentiate to generate all other cells that comprise a tumor. B) Clonal evolution model whereby any cell can initiate a tumor, and through genetic mutations, establish intratumoral heterogeneity. These cells may also possess self-renewing capacity which was obtained through stochastic processes. *Lightening bolts represent mutagenesis and asterisks represent mutations.* Taken from (Campbell & Polyak, 2007).

In the following thesis, intratumoral heterogeneity within the context of ovarian cancer will be explored, as well as the applicability of the cancer stem cell model in describing this heterogeneity.
Specific Aims

Specific aims of the following thesis include:

1. Comprehensive review of literature to characterize the nature and extent of intratumoral heterogeneity found in ovarian cancer.

2. Investigation into the current evidence for the ovarian cancer stem cells.

3. Conclusion on the validity of the cancer stem cell model in predicting intratumoral heterogeneity in ovarian cancer.
INTRATUMORAL HETEROGENEITY IN OVARIAN CANCER

Ovarian cancer is not a single disease, but includes several subtypes which range from relatively benign to aggressive, metastatic tumors. These subtypes vary greatly in morphology, genetic alterations, and interestingly, the proposed cell of origin, which in most cases is theorized to be from tissues outside of the ovary. In the following section will outline the ovarian cancer subtypes and discuss their proposed pathogenesis. Next, we will focus on intratumoral heterogeneity within ovarian cancer, specifically, how the genetic and epigenetic alterations, microenvironment, and cell plasticity contribute to the remarkable diversity of cancer cell populations typically found in this disease.

Ovarian Cancer Subtypes and Pathogenesis

Ovarian cancer is not a single disease, but a grouping of cancers which have differing behaviors and morphologies, cells of origin and responses to treatment. Beyond the histological appearance of the cells (Figure 5), each subtype differs in its clinical course and genetic expression profile, although much overlap has been known to occur. The characteristics of each subtype of ovarian cancer are summarized in Table 2.

The five most common forms of ovarian cancer are very diverse, yet high grade serous carcinoma research dominates the literature, presumably due to its aggressive nature and high frequency. An attempt was made to reclassify
ovarian cancer into only two types, ‘Type I and Type II’ (Kurman & Shih, 2010), however this approach has been criticized for lumping together the more rare subtypes of ovarian cancer and thus hindering our understanding and progress toward managing these important diseases (McCluggage, 2011; Prat, 2012). Although rare, the clear cell carcinoma subtype is actually more deadly when diagnosed in the late stages than the common HGSC, because of its poor response to chemotherapy (Prat, 2012). The study of this particular subtype of cancer is made difficult by its rarity, however recognition of it as a distinct form has advantages for a much needed focused study. For a detailed outline of each subtype see Appendix I.
Figure 5. Ovarian Cancer Subtypes. A) High-Grade Serous Carcinoma (HGSC); B) Low-Grade Serous Carcinoma (LGSC); C) Mucinous Carcinoma (MC); D) Endometrioid Carcinoma (EC); E) Clear Cell Carcinoma (CCC)
Table 2. Ovarian Cancer Subtypes. Summary of distinguishing features of five ovarian cancer subtypes, which account for nearly 98% of all ovarian cancer manifestations. HGSC – High Grade Serous Carcinoma; LGSC – Low Grade Serous Carcinoma; MC – Mucinous Carcinoma; EC – Endometrioid Carcinoma; CCC – Clear Cell Carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>HGSC</th>
<th>LGSC</th>
<th>MC</th>
<th>EC</th>
<th>CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>68-71%</td>
<td>&lt;5%</td>
<td>3-4%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Aggressive</td>
<td>Mostly benign</td>
<td>Mostly benign</td>
<td>Very benign</td>
<td>Benign if detected early, aggressive if late stage</td>
</tr>
<tr>
<td>Common genetic signature and expression profile</td>
<td>TP53, BRCA, WT1, p16, ER, Ki67</td>
<td>Similar expression profile to HGSC, except low Ki67. May contain BRAF and KRAS mutations.</td>
<td>CDX2, KRAS, CK7</td>
<td>HNPCC, PTEN, ARID1A, CTNNB1, ER</td>
<td>HNF1-beta, ARID1A, ARHGDIG</td>
</tr>
<tr>
<td>Cell of origin</td>
<td>Fallopian tube or ovarian surface epithelium</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Endometrium or myometrium</td>
<td>Endometrium or myometrium</td>
</tr>
<tr>
<td>Response to therapy</td>
<td>Initially sensitive to chemotherapy but develops resistance and recurs</td>
<td>Unknown, preliminary data suggests insensitivity</td>
<td>Unknown</td>
<td>Sensitive to chemotherapy</td>
<td>Typically unresponsive to treatment</td>
</tr>
</tbody>
</table>

The pathogenesis of ovarian cancer including the cell of origin is not yet understood and is believed by some to be the missing piece of a puzzle that would allow for early detection and effective treatment (Kurman & Shih, 2010; Prat, 2012). Determining the cell of origin requires identification of a precursor condition, before the tumor overruns the tissue and obscures the initiation site.
Tubal intraepithelial carcinoma, endometriosis, cysts or borderline tumors (Figure 6) have been postulated as precursor lesions to development of cancer. Often in ovarian cancer, the disease has advanced well beyond this stage at the time of presentation, making it difficult to determine where the abnormality started.

The first study that was able to identify consistent precursor lesions in the fallopian tube came out of careful examination of specimens obtained from prophylactic salpingo-oophorectomies in women who were positive for BRCA. This provided evidence for HGSC cell of origin in the fimbriae for patients with hereditary BRCA mutations (Kindelberger et al., 2007). Evidence continues to mount in support of the notion that the precursor lesion for ovarian cancer is serous tubal intraepithelial carcinoma, (serous TIC or STIC) occurring in the distal fallopian tube, however, ovarian surface epithelium cannot be ruled out entirely as a potential site of origin (Bowtell, 2010; Kurman, 2013), and it seems likely that both regions may be involved in a case-by-case manner. Illustrations of the various pathways hypothesized in the early development of ovarian cancer subtypes are presented in an influential paper by Kurman & Shih in 2010 (Figure 7). Non-serous subtypes of ovarian cancer, such as endometrioid and clear cell carcinoma, are also postulated to originate from tissues outside of the ovary, most likely the endometrium. Mucinous carcinoma of the ovary has been postulated to originate in the endocervix and other areas, but compelling
evidence is lacking for this subtype as well. (Dubeau & Drapkin, 2013). Research on these subtypes is limited by the relative rarity of the disease.
Figure 6. Hypothesized Progression from Site of Origin in Serous Ovarian Carcinoma. Precancerous lesions develop from abnormal cells in the fallopian tube or ovarian surface epithelium. Progression involves formation of various precancerous lesions, and in the case of TIC, shedding of malignant cells into the peritoneal cavity. Taken from (McCluggage, 2011).
Figure 7. Pathogenesis of Ovarian Cancer Subtypes from Hypothesized Sites of Origin. A) Cells shed from fallopian tube fimbriae generate inclusion cysts on the ovary, which can progress to high or low-grade serous carcinoma with mutations in TP53 or KRAS/BRAF respectively. Low grade serous carcinoma is often preceded by serous borderline tumor (SBT). HGSC can also develop from STIC shed from the fimbriae. Occasionally, HGSC can develop from LGSC. B) Both endometroid and clear cell carcinoma of the ovary develop from retrograde menstruation as a result of endometriosis. Borderline tumors develop as an intermediate step in this process. Adapted from (Kurman & Shih, 2010).
Despite numerous studies to determine the true origins of ovarian cancer, there remains conflicting evidence and many unanswered questions. It should not be overlooked that some ovarian tumors present as heterogeneous mixtures of various histological phenotypes, and currently there is no evidence that might explain this pathogenesis. Furthermore, although the genetic signatures of these various subtypes have been more or less defined, it’s crucial to keep in mind the fact that these signatures are not all inclusive – and there is much variability within subtypes, and overlap between them. For example, high grade serous carcinoma subtypes have been characterized as different from other types of ovarian cancer for the absence of PI3K pathway involvement, but more recent data suggests this pathway is more closely linked to HGSC than previously thought (Prat, 2012). The same pathway has been suggested as a characteristic of endometriod carcinoma, yet PTEN mutations have been shown to occur in only 20% of cases (Prat, 2012). Thus, the variability within subtypes and the overlapping nature of their genetic signatures is apparent, and begs the question of whether our classification system is misleading research efforts and misinforming treatment strategies.

The possibility that all ovarian cancer subtypes might share the same cell of origin has been suggested in support of the cancer stem cell theory (Shah & Landen, 2014). Considering the fact that ovarian cancer can present as heterogeneous mixtures of the various subtypes, and the overlapping nature of their genetic signatures, further work is warranted to explore this idea. In the next
section, a detailed review of the heterogeneity existing in ovarian cancer genetics and epigenetics will be given. Until a consensus is reached on the true cell(s) of origin, however, our understanding of how intratumoral heterogeneity arises will remain incomplete, as will our ability to effectively treat this disease.

**Genetic, Epigenetic and Expression Profiles**

There are a variety of ways in which a cancer genome can be altered, leading to changes in protein function or expression levels. Somatic mutations include changes in DNA sequence such as point mutations, where one base substitutes for another; insertions or deletions of parts of the genome; rearrangements within the genome; and amplification, whereby a normal diploid gene which normally has only two copies can have up to several hundred copies (also termed ‘somatic copy number alterations’ or SCNA). Germline mutations, such as BRCA1/2, are associated with increased risk for certain cancers such as ovarian and breast, and are not the result of random mutations, but represent permanent, hereditary alterations in the genome. In cancer, somatic and germline alterations affect the ability of a cancer cell to grow and divide independent of growth factors and other regulatory signaling thus obtaining one of the most important hallmarks of cancer: chronic proliferation (Hanahan & Weinberg, 2011).

In addition to genetic mutations, the impact of epigenetic alterations – modifications to the genome that affect transcription but do not involve sequence alteration – is emerging as a crucial component of tumor progression and
heterogeneity. Prominently studied epigenetic factors include hyper/hypo-methylation of gene promoters and histone acetylation and methylation, both of which affect DNA architecture resulting in increases or decreases in gene transcription. Hypermethylation of the BRCA1 promoter, for example, was discovered in a significant portion of sporadic breast and ovarian tumors, indicating the role of the BRCA1/2 gene beyond traditional hereditary mutations (Esteller et al., 2000).

Of course as a consequence of genetic and epigenetic alterations, mRNA and, of growing importance, microRNA (Voorhoeve, 2010) expression are often found to be abnormal in cancer cells. Analyses of expression profiles may provide additional insight beyond transcriptional processes.

Attempts to define and characterize the genetic, epigenetic and expression landscape of ovarian cancer have revealed insights into the nature of heterogeneity in ovarian cancer. The largest study to date involved a massive collaboration by the Cancer Genome Atlas Research Network, a branch of the National Institutes of Health, to sequence the coding exons of the genome (exome) of 316 patient samples of serous ovarian cystadenocarcinoma, as well as analysis of mRNA and microRNA expression, promoter methylation and DNA copy number in 489 samples (Cancer Genome Atlas Research Network, 2011). Although this study was restricted to high grade serous carcinoma (HGSC), it provided strong support for the role of the TP53 oncogene in this subtype, as
previously reported (Ahmed et al., 2010). From these data, it appears that mutations in TP53 are present in 96-100% of HGSC, and represents the only universally shared genetic alteration of any ovarian cancer subtype. Another significant finding of the Cancer Genome Atlas and Research Network was the alterations in BRCA1/2 gene present in 33% of HGSC cases (including germline, somatic and epigenetic silencing). The number of recurrent genetic mutations found by this study was low, including only nine identified genes (Table 3); other analyses identified 122 missense mutations which were potentially oncogenic but not necessarily recurrent, indicating the considerable heterogeneity of genetic mutations in HGSC.

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of mutations</th>
<th>No. validated</th>
<th>No. unvalidated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>302</td>
<td>294</td>
<td>8</td>
</tr>
<tr>
<td>BRCA1</td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>CSMD3</td>
<td>19</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>NF1</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>CDK12</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>FAT3</td>
<td>19</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>GABRA6</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>BRCA2</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>RB1</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Recurrent Genetic Mutations Found in HGSC. Taken from (Cancer Genome Atlas Research Network, 2011).

Copy number analysis, in contrast to genetic mutations, revealed a large number of somatic copy number alterations (SCNAs), many of which were recurrent in 20-50% of tumors (Cancer Genome Atlas Research Network, 2011). Notable
examples of SCNAs found were focal amplifications in CCNE1, MYC and MECOM, and focal deletions in PTEN, RB1 and NF1. These genes are implicated in various signaling pathways, notably RB signaling, PI3K (phosphoinositide 3-kinase)-Ras, (mitogen-activated protein kinase- extracellular signal-regulated kinases (MAPK-ERK) and transforming growth factor beta (TGF-β), all of which control some aspect of cell cycle progression, proliferation and survival. These data show that there are more commonalities within SNCAs among HGSC tumors, and therefore represent a less heterogenous factor in this disease than genetic mutations. It was postulated in the report that perhaps the prevalence of mutations in DNA repair genes contributed to the high number of SCNAs.

Based on the combined analyses of genetic mutations, copy number variation and epigenetic changes, the Cancer Genome Atlas Research Network identified four important signaling pathways in HGSC: RB1, PI3K/RAS, NOTCH and FOXM1, with FOXM1 being altered in a significant majority of cases (87%), followed by RB1 (67%); and PI3K/RAS (45%) and NOTCH (22%) being less representative (Cancer Genome Atlas Research Network, 2011). Thus although there is remarkable intertumoral heterogeneity of alterations in the various factors involved in these pathways, there is less heterogeneity in which pathways are involved in HGSC. From the perspective of drug treatment, knowing which pathways to target in order to have a large impact on the majority of HGSC cases
will have important implications, even if the factors that cause the deregulation vary widely.

In contrast to HGSC, low grade serous carcinoma (LGSC) does not involve p53 mutations and instead is associated with mutations in either KRAS or BRAF (Ho, Kurman, Dehari, Wang, & Shih, 2004; Singer et al., 2003). The difference in signaling pathway involvement has been suggested to indicate that these two forms of serous carcinoma evolve by separate mechanisms, and that LGSC is not an “intermediate step” toward development of HGSC (Russell & McCluggage, 2004). A comparison of the pathways involving p53, KRAS and BRAF are illustrated in Figure 8. Of note, there appears to be significant opportunity for cross-talk between these two pathways.

Akt plays a central role in the PI3K pathway and cross-talks with other important pathways in cancer biology. In addition to HGSC, clear cell carcinoma has also been shown to exhibit activating mutations in the PI3K pathway, specifically mutations in the PI3KCA gene itself, which resulted in intense phosphorylated Akt activity (Kuo et al., 2009). Mucinous carcinoma exhibits relatively frequent mutations in the KRAS gene, similar to LGSC (Naik, Seligmann, & Perren, 2012; Xiong et al., 2013).
Figure 8. Ras-Raf and PI3K-AKT Signaling Pathways. Dimerization of receptor tyrosine kinase EGFR (Epidermal growth factor receptor) occurs upon ligand binding of TGF-α or EGF. Subsequent recruitment of Grb2 and other signaling molecules result in a cascade of events which lead to cell proliferation, growth, and anti-apoptotic effects. Mutations found in ovarian cancer include decrease or loss of p53, PTEN and CTNNB1, and increased levels of Ras, Raf, PI3K, Akt and RB1. Figure taken from (Jingxian Zhang et al., 2012).
Endometrioid carcinoma is the only ovarian cancer subtype to show a relatively high frequency of mutation in the CTNNB1 gene, which encodes a protein beta-catenin that is involved in the regulation of cell growth, proliferation and apoptosis, as well as the epithelial to mesenchymal transition (EMT) (Arend, Londoño-Joshi, Straughn, & Buchsbaum, 2013; Lech et al., 2013). Beta-catenin is a key component of the Wnt signaling pathway (Figure 9), and when sufficient unphosphorylated amounts accumulate in the cell cytoplasm, this protein migrates to the nucleus to affect gene transcription. Phosphorylation of beta-catenin by casein kinase 1 (CK1) and GSKb (glycogen synthase kinase) results in its ubiquitination and proteosomal degradation.

Although the CTNNB1 mutation is not typically found in the other subtypes, other alterations can similarly lead to the dysregulation of the Wnt pathway and are thought to contribute to pathogenesis in these subtypes (Arend et al., 2013). Recall from Figure 8 that the PI3K/Akt/mTOR pathway crosstalks with the CTNNB1 pathway through inhibition of GSK3b, thus offering an opportunity for crosstalk and indirect activation of the Wnt pathway.

In addition to cell proliferation, survival and migration, the Wnt pathway is an important control mechanism in stem cell self-renewal (Pardal, Clarke, & Morrison, 2003). This connection will be explored in more detail in a later section of this thesis.
Figure 9. Wnt Signaling Pathway. A) WNT ligands are not bound to the receptor Frizzled, beta-catenin is phosphorylated and degraded, ensuring repression of gene transcription by co-repressor groucho. B) WNT ligand binds the Frizzled and LRP5/6 receptor complex, preventing phosphorylation of beta-catenin by GSK3b and CK1. Beta-catenin accumulates in cell cytoplasm and moves to nucleus, where it displaces groucho and forms a transcriptional complex with TCF/LEF family proteins, BCL9/LGS, and Pygo, and promotes transcription of genes involved in cell proliferation and survival. Figure taken from (Arend et al., 2013).
Endometriod and clear cell carcinoma (EC and CCC, respectively), do not show alterations in p53, KRAS or BRCA, however have recently been shown to harbor frequent mutations in gene ARID1A, with the mutation occurring in 46-57% of clear cell carcinoma and 30% of endometriod carcinoma (Jones et al., 2010; Wiegand et al., 2010; Wu, Wang, & Shih, 2014). ARID1A codes for a protein, BAF250A, which is a critical component of the ATP-dependent chromatin remodeling complex, SWI/SNF (see Figure 10). The SWI/SNF complex regulates chromatin remodeling and gene transcription, specifically of genes involved in repressing the cell cycle, and inhibiting stem cell self-renewal, thus loss of this protein would result in increased proliferation and maintenance of the stem cell state (Reisman, Giarros, & Thompson, 2009).

**Figure 10. The SWF/SNF complex including BAF250A.** This complex uses the energy of ATP to rearrange nucleosomes and alter the accessibility of DNA for transcription and other processes. Mutations in ARID1A are found frequently in endometriod and clear cell carcinoma, and result in loss of BAF250A protein necessary to form these complexes. The SWF/SNF complex shown is involved in cell proliferation and differentiation. Illustration taken from (L. Ho & Crabtree, 2010).
The role of ARID1A is still being explored, and mounting evidence suggests it extends far beyond direct regulation of gene transcription, but also is involved in DNA repair mechanisms, exhibits co-dependency with p53, and collaborates with dysregulation of the PI3K/Akt signaling pathway in tumorigenesis (Wu et al., 2014). Thus, although these subtypes of cancer do not share the BRCA or p53 mutations common in high grade serous carcinoma, some similarities in dysregulation of DNA repair and apoptosis are observed through the mutation of ARID1A and loss of BAF250A.

In examining the genetic mutations involved in the various subtypes of ovarian cancer, the list is actually quite short, involving common oncogenes and tumor suppressors – p53, KRAS, RAF, PTEN, BRCA – as well as less common mutations in CTNNB1 and ARID1A. Of greater significance within individual subtypes seems to be the alterations in cell signaling pathways which do not involve mutations but rather somatic copy number alterations and epigenetic changes. These alterations confer remarkable intertumoral heterogeneity in molecular profiles within various subtypes of ovarian cancer. Despite this heterogeneity, however, the signaling pathways affected by it also comprise a short list – with the Ras/raf/mek and PI3K/Akt/MAPK dominating current research, but emerging roles for the Wnt pathway and others are also on the horizon. Cross-talk between these various pathways is an intriguing question which deserves further investigation, so as to draw connections and possible insight into the pathogenesis of distinct ovarian cancer subtypes, and to predict
potential routes of resistance to molecular targeted therapies in order to design more effective drugs.

**Microenvironment**

The importance of the microenvironment in tumor progression is gaining increasing recognition for virtually all cancer types (Kalluri & Zeisberg, 2006; Liotta & Kohn, 2001; Tlsty & Coussens, 2006). The tumor microenvironment is a heterogeneous mixture of stromal cells that co-evolve with the cancer itself, becoming transformed to support tumor growth (Figure 11) (Briest et al., 2012). Specifically, endothelial cells, macrophages and fibroblasts have been shown to play a critical role in tumor pathogenesis by inducing angiogenesis and remodeling the extracellular matrix, as well as by secreting key cytokines that aid in tumor metastasis. The tumor stroma has also been implicated in drug resistance (Junttila & de Sauvage, 2013)
Figure 11. The cells of the heterogeneous tumor microenvironment. Taken from (Junttila & de Sauvage, 2013)

Ovarian cancer cells have been shown to signal stromal cells for inducing angiogenesis as well as the inflammatory response, both of which aid in cancer progression (Gavalas et al., 2013; Shan & Liu, 2009). Ovarian cancer is unique among other cancers for its tumor microenvironment, the intraperitoneal space, which contains a multitude of organs covered in mesothelium, as well as the fatty omentum, and in addition, permits cancer cells to exist suspended in fluidic ascites in late stage disease (Naora, 2014). Tumor-stromal interactions are
mediated by a variety of cell surface molecules and cytokine signaling (Figure 12); for example mesothelial attachment is known to be mediated in part by CA125-mesothelin, CD44HA, and various integrins (Naora, 2014). Attempts to target these molecules and hinder tumor attachment of ovarian cells to the mesothelium have largely failed due to the heterogeneous expression of particular molecules within the same disease (Cannistra et al., 1993; Heyman et al., 2008).

Angiogenesis is initiated and controlled through cytokine signaling of the ovarian cancer cell itself as well as activated stromal cells, which secrete various factors including VEGF, FGF-2, IL-6, IL-8, angiopoietin, and PDGF (Martin & Schilder, 2007).

Adipocytes are important sources of nutrients and promote proliferation of ovarian cancer cells through direct transfer of lipids to the cells (Nieman et al., 2011).

A matter of intense research is that ovarian cancer cells induce transformations that result in generation of cancer associated fibroblasts (CAFs), which have numerous functions to enhance tumor progression (Kalluri & Zeisberg, 2006). CAFs arise from a variety of sources, including resident fibroblasts and mesenchymal stem cells which have been recruited from bone marrow or adipose tissue (Y. Zhang et al., 2011).
Figure 12. Interaction pathways in the ovarian cancer microenvironment. Ovarian cancer cells suspended in the peritoneal cavity interact and attach to mesothelium, and receive proliferative support from adipocytes. Ovarian cell signaling recruits macrophage and fibroblast progenitors to the site of implantation, and induces their transformation into “cancer associated” support cells. Influence on immune cell reactivity is also mediated by tumor cell signaling as well as cancer associated stromal cells. TAM – tumor associated macrophage; CAF – cancer associated fibroblast. Taken from (Naora, 2014).

Transformation of cells into CAFs is mediated by TGF-β signaling from the ovarian cancer cells (Ko et al., 2012), and are important for tumor growth and proliferation, as well as metastasis. They have been further implicated in tumor initiation, although the exact mechanisms of these processes are not yet understood (Kalluri & Zeisberg, 2006).

Tumor-associated macrophages (TAMs) are abundant in ovarian cancer ascites are known to promote tumor progression by inhibiting the adaptive immune system through expression of immunosuppressive cytokines such as interleukin (IL)-10, TGF-β, CCL17, CCL18, and CCL22 (Naora, 2014; Yigit, Massuger, Figdor, & Torensma, 2010). In addition, TAMs are known to promote tumor invasion and metastasis (Quail & Joyce, 2013), and specific studies have confirmed this link in ovarian cancer mouse models (Neyen et al., 2013).

In summary, the tumor microenvironment is both remarkably heterogeneous and functionally influential aspect of cancer biology, and is closely associated with tumor initiation, progression and metastasis. The intimate cross-talk between the
tumor and stromal cells is a large area of research that promises new avenues for targeted therapy.

**Differentiation and Cell Plasticity**

Of recently intensified interest in the study of intratumoral heterogeneity is the ability of cancer cells to undergo epigenetic changes which influence the degree to which they display an epithelial or mesenchymal phenotype. The epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are programs thought to underlie the ability of cancer cells to develop all the necessary ‘hallmarks’ required for metastasis: whereby certain cancer cells escape the confines of the tumor, enter the vasculature and travel to distant sites where they then exit the vasculature and set up new colonies (Figure 13).

Until recently, while it was recognized that metastasis is associated with loss of cell adhesion molecules such as E-cadherin, N-cadherin and shifts in integrin expression, the mechanism was poorly understood (Hanahan & Weinberg, 2000). Current research has implicated EMT as the driver behind metastasis, certain transcription factors, named Snail, Slug, Twist, and Zeb1/2, have been shown to repress the epithelial phenotype when activated and thus play a critical role in cell plasticity. Beyond EMT, less is known about the reverse process, which involves extravasation and seeding in a new microenvironment, apparently involving MET. Research has been directed toward cancer stem cells as playing a role in this process, especially in light of their tumorigenic properties (Scheel &
Weinberg, 2012). Connections between cancer stem cells, the EMT/MET programs and metastasis will be revisited in a later section of this thesis.

Figure 13. Key steps in tumor metastasis involve EMT and MET programs. A select subpopulation of cancer cells obtain a mesenchymal phenotype which allows them to migrate away from the primary tumor, intravasate, circulate to distant sites, extravasate, and undergo MET to generate a new colony with the differentiated epithelial phenotype of the original tumor. Taken from (Scheel & Weinberg, 2012)
A Review of Stem Cell Biology

A stem cell is a multi, pluri, or toti-potent cell capable of differentiating into various more specialized cells. Totipotent stem cells have the greatest plasticity, or ability to differentiate into the most versatile progeny, while the multipotent stem cell has the least plasticity, or limited paths of differentiation (Yamanaka & Ralston, 2010). Totipotent stem cells are only found during embryogenesis. In addition to producing differentiated progeny, stem cells are capable of self-renewal, and their actions are very carefully regulated through cell-cell and cell-matrix interactions, as well as through various signaling molecules.

During embryogenesis, stem cells generate all tissues of the body. Determination of cell fate involves specific genes and transcription factors, however, other mechanisms that involve position, polarization, shape, signaling and division plane probably play a greater role in the earlier stages of lineage specification (Yamanaka & Ralston, 2010). By extension, in order for cell position to play a role in fate, cells must have a sense of polarity and spatial awareness brought about by cell-cell contacts.

Differential expression of transcription factors leads to further specialization into inner cell mass (future fetus and yolk sac) and trophoblast layer (placental tissue)
during embryogenesis. These factors and their roles are summarized in Table 4 (Appendix II).

At the heart of embryonic stem cell regulation are the transcription factors Oct4, Sox2 and Nanog, which are responsible for maintaining pluripotency and self-renewal capabilities. Their activation is mediated through various signaling pathways, as shown in Figure 14.

**Figure 14. Regulatory Pathways in Embryonic Stem Cells.** Both LIF-Stat and BMP pathways are essential to embryonic stem cell self-renewal in the mouse, but LIF-JAK-STAT is dispensable in human. JAK-STAT pathway activates KLF4 (not shown) which activates Sox2. FGF-MEK pathway (not shown) initiates differentiation. PI3-kinase (not shown) activates Tcf3 through LIF. Activin/Nodal
TGFβ and FGF are required for human embryonic stem cells. Wnt pathway signaling is required for both mouse and human embryonic stem cell self-renewal. Figure taken from (Heng & Ng, 2010).

Various tissue-specific adult stem cells (referred to from here as ‘stem cells’) have been identified. Perhaps the most extensively studied are hematopoietic stem cells (HSCs), which reside in bone marrow and can differentiate into any of the various blood cells (Figure 15). HSCs follow a strict hierarchy where each division gives rise to a more differentiated cell, one that is committed to a particular phenotype.
Figure 15. Hematopoietic Stem Cells. Multipotent stem cells can self-renew and give rise to multipotent progenitors. These progenitors will then differentiate and commit to a particular cell lineage, meanwhile the self-renewal and multipotency is lost. Taken from (Ema, Kobayashi, & Nakauchi, 2010).

Mesenchymal stem cells (MSCs) are another type of adult stem cell that can differentiate into a variety of tissues, including adipocytes, chondrocytes, myocytes, fibroblasts, neurons, osteocytes and epithelial cells. The majority of MSCs reside primarily in bone marrow, and can be recruited through cell signaling molecules to home to certain tissues and differentiate when needed (Zhuge, Liu, & Velazquez, 2010).

Stem cells are known to localize to a specialized microenvironment called a ‘niche,’ in which they receive extrinsic regulatory signals through cell-cell and cell-matrix connections, hormones, and other signaling molecules. These signals regulate stem cell behavior, the most crucial component being the decision to self-renew or differentiate. If the stem cell leaves its niche, it will differentiate by default, indicating the signals involved in the niche help to maintain the stem cell state (Ferraro, Celso, & Scadden, 2010).

Thus, the balance of stem cell supply to differentiated tissue is controlled by a host of mechanisms, including cell-cell contact between stem cells and their progeny. In contrast to the above example, if a mature, differentiated cell loses contact with the stem cell base layer, it can revert to a stem cell, or de-differentiate. This was demonstrated in recent experiments by Tata et al, where
laser ablation of the stem cell basal layer in the airway epithelium caused mature
surrounding cells to proliferate and some de-differentiated to restore the stem cell
population. In vitro experiments revealed the mechanism which normally
prohibited this de-differentiation was cell-cell contact. Moreover, it was shown
that depending on the degree of maturity, certain cells were more capable of de-
differentiation than others, pointing to a spectrum of transitional changes from
one phenotype to the other (Tata et al., 2013).

In response to extrinsic signaling, various intracellular signaling pathways are
activated. Of particular interest in cancer research are the pathways involved in
stem cell self-renewal, namely Notch, Wnt, Hedgehog and TGF-b. These
pathways are illustrated below.

**Notch Pathway**

The Notch pathway has been implicated in regulating cell-cell communication –
as indicated in Figure 16 by the neighboring cell, the Notch-Jagged connection is
made between two cells of close proximity, and regulates stem cell self-renewal.
Overexpression of Notch signaling molecules has been noted in certain cancers
including ovarian, and has been linked to platinum resistance (McAuliffe et al.,
2012). Drugs which target gamma-secretase have been developed (GSIs) and
have been shown to eliminate cancer stem cell populations and restore
chemosensitivity (McAuliffe et al., 2012)
Figure 16. The Notch Pathway. Upon activation of the Notch receptor by the ligand ‘Jagged,’ two proteases are activated – extracellular ADAM proteinase and intracellular gamma-secretase. The intracellular fragment of Notch (ICN) falls off the receptor as a result and translocates to the nucleus to dislodge a corepressor (CoR) and initiate transcription of Notch target genes such as Hes1. Taken from (Heidel, Mar, & Armstrong, 2011)

Wnt Pathway

The Wnt pathway is involved in regulation of stem cell self-renewal, proliferation, differentiation, and apoptosis by activation of transcription through its central mediator, b-catenin, as shown in Figure 17. In addition, it is thought to be a major signaling pathway involved in epithelial-mesenchymal transition (EMT) necessary for tumor invasion and metastases (Neth et al., 2007). Dysregulation of the Wnt pathway has been strongly associated with several cancers including
ovarian, where it has been shown to affect tumorigenesis and progression (Arend et al., 2013).

Figure 17. Wnt Signaling Pathway. In the absence of Wnt signaling, the b-catenin molecule is associated with APC, GSK-3 and axin. In this complex, b-catenin is phosphorylated by GSK-3 and CK1, and is thus marked for proteosomal degradation. Gene transcription does not occur, as the TCF/LEF transcription factor is bound and inhibited by Groucho. When Wnt binds to its receptor, Frizzled, b-catenin is released from the inhibitory complex and able to translocate to the nucleus, dislodge Groucho and initiate gene transcription. Taken from (Heidel et al., 2011).

Hedgehog pathway

The Hedgehog pathway is involved in maintaining cell polarity, and the balance between stem cell supply and demand through regulation of proliferation and
differentiation. Dysregulation of this pathway has been correlated with basal cell carcinoma among other cancers, and the FDA approved drug vismodegib has been used to treat BCC by antagonizing the receptor Smoothened (SMO), shown below in Figure 18.

![The Hedgehog Pathway](image)

**Figure 18. The Hedgehog Pathway.** In the absence of Hh, the transmembrane receptor Patched (Ptch) inhibits another receptor, smoothened (Smo). Upon binding of Hh to Ptch, it releases the inhibition on Smo, and Gli transcription factors are released from Smo to initiate gene transcription. Taken from (Heidel et al., 2011).

**TGF-β pathway**

TGF-beta signaling is initiated by a large number of different ligands, and makes up the TGF-β superfamily (Figure 19). Ligands include the canonical TGF-βs, of which there are three, Nodal, and bone morphogenic protein (BMPs). These
pathways are critical during embryogenesis as well as in maintaining self-renewal and senescence in adult stem cells (Gaarenstroom & Hill, 2014).

**Figure 19. TGF-beta superfamily signaling pathways.** TGF-b ligands are held by latency associated peptides (LAPs) and bound in the extracellular matrix (ECM) until they are released by various mechanisms (dashed arrow). TGF-b can then bind to a variety of Type I and Type II serine-threonine kinases. Subsequent phosphorylation of R-Smads occurs, and there are two major divisions which vary based on the ligand/receptor. Inhibitory Smads6 inhibits only one branch of the signaling pathway, while Smad7 can inhibit both. Phosphorylated Smads join with co-Smad to translocate to nucleus and regulate gene transcription. Figure taken from (Oshimori & Fuchs, 2012).
**Stem Cells and Cancer**

Tumors have been conceptualized as an “aberrant organ” which originates, as in normal development, from a multipotent stem cell, capable of differentiating into a wide range of heterogeneous cell types; except in the case of cancer, the initiating cell has undergone a cancerous transformation which allows it to proliferate in the absence of regulatory controls (Reya, Morrison, Clarke, & Weissman, 2001). The classical example of this process is found in teratocarcinoma, where multiple differentiated tissues types such as cartilage and bone coexist within a single tumor (Figure 20), indicating the involvement of a progenitor cell (a human mesenchymal stem cell) which differentiated abnormally into various tissue types independent of external queues. Other cancers, including breast and ovarian, have also shown heterogeneously differentiated cell populations, and these findings fuel questions about the role of stem cells in generating tumors (Abelson et al., 2012; Smart et al., 2013). If cancer stem cells do exist, and tumorigenic cells can differentiate into the remarkably heterogeneous populations comprising a tumor, this could have wide-reaching implications for new targeted drug therapies. The following section will highlight some of the connections found between normal stem cell behavior and the nature of tumor progression.
Figure 20. Metastatic Teratocarcinoma. Case study of a 26-year-old patient with known teratocarcinoma of the testicle was found in autopsy to have cartilage deposits in the left anterior descending coronary artery which led to cardiac arrest and death of the patient. Cartilage deposits were also found in the lung. Both were a result of metastasis of the teratocarcinoma. Taken from (Nath, Bhattacharya, & Bharadwaj, 2011).

Stem cells and cancer cells may have similar patterns of division (Figure 21) which illustrate a few common traits. A key feature shared between stem cells and some cancer cells is self-renewal – a complex and necessary function which involves not only the ability to divide asymmetrically, but to balance the supply of stem cells with the amount of daughter cells. This process is highly regulated in normal stem cells and involves the pathways described in the previous section. When these pathways become dysregulated, aberrant self-renewal, proliferation,
and independence from external signaling can result in the development of cancer. For example, mutations that inappropriately activate the Wnt and Hedgehog pathways have been shown to cause colorectal cancer and basal cell carcinoma, respectively (Taipale & Beachy, 2001). Dysregulation of neural stem cell signaling pathways also leads to cancer; when pathways which regulate self-renewal are constitutively active it has been shown to cause primary glioblastoma multiforme (Zhu & Parada, 2002). Turning off these pathways, in converse, tends to reverse cancer progression (Liu, Albrecht, Ni, Yang, & Li, 2013). These findings suggest the same pathways that regulate self-renewal processes in normal stem cells are important in tumorigenesis and cancer progression.

Another feature common to stem cells and cancer is differentiation into daughter cells with reduced proliferative potential – this hierarchy lies at the core of the CSC theory. A number of studies in which isolated subpopulations of cancer cells were transplanted into immunodeficient mice found that only a small subset of cells were capable of generating tumors, and these tumors recapitulated the heterogeneous parental disease, including phenotypes with limited proliferative potential (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Lapidot et al., 1994; Singh et al., 2003). Thus, tumor populations consist of a variety of phenotypes with differing tumorigenic potential, similar to the stratification in
normal tissues, where undifferentiated stem cells co-exist with their differentiated daughter cells.

**Figure 21. Normal Stem Cell Vs. Cancer Stem Cell.** a) Totipotent embryonic stem cells develop into different lineages of stem cells that are self-renewing and give rise to all tissues in the body. b) If normal stem cells undergo a certain number of mutations they can become cancerous while retaining self-renewing properties. Alternatively, a mature cell may de-differentiate into a stem cell and acquire self-renewing abilities, at the same time, acquiring other mutations that
transform the cell into a malignant cancer stem cell. Figure taken from (Pardal et al., 2003).

The first example of a “cancer stem cell” was found in human acute myeloid leukemia (AML). In 1994, Lapidot et al reported the discovery that a particular cell phenotype, CD34+/CD38-, could recapitulate all features of AML disease in mice upon transplantation, while CD34+/CD38+ and CD34- fractions could not (Lapidot et al., 1994). These and subsequent findings strongly support a hierarchical organization whereby a single primitive stem cell gives rise to all subpopulations of cells, which possess remarkable heterogeneity (Bonnet & Dick, 1997). Other examples where cancer stem cells have been identified include breast cancer and glioblastoma, and the work that has spanned over a decade has been summarized in reviews (Badve & Nakshatri, 2012; Dontu, Liu, & Wicha, 2005; Stopschinski, Beier, & Beier, 2013). In each case, similar to the AML model, cancer stem cells are identified based on their ability to initiate tumors and recapitulate the heterogeneous disease in vivo upon injection into immunocompromised mice. Cell surface markers, namely CD24-/CD44+ in breast and CD133+ in glioma cells, were identified as markers of their respective cancer stem cells.

In contrast to AML and other cancers, a study on melanoma revealed that a large percentage, between 25-27%, of cancer cells isolated from primary and metastatic sites are capable of initiating a tumor in severely immunocompromised mice (Quintana et al., 2008). These findings conflicted with
previous results in mice with more in-tact immune systems, and brought attention to the possibility that xenotransplantation assays may underestimate the tumorigeneicy of cancer cells in other studies because of a remnant immune response. It also suggested that some cancers such as melanoma may not follow the stem cell model, or that they may differ on the extent to which cancer stem cells are involved.

Although the identification and characterization of cancer stem cells in AML, breast cancer and glioblastoma have been intensively studied, the existence of ovarian cancer stem cells is a matter of heated debate. One aspect that is complicating the study of ovarian cancer stem cells is lack of consensus on the true cell of origin – fallopian tubal epithelium vs ovarian surface epithelium. Yet even in organ systems which have been robustly characterized in terms of stem cells and their niche, such as in the intestinal crypt (Barker, 2014), controversy ensues on the reliability of cell surface markers in detecting and identifying tumorigenic cells. Advanced techniques such as lineage tracing are now allowing scientists to study longitudinal stem cell behavior and function in vivo (Blanpain & Simons, 2013). Recent findings on new markers such as Lgr5 using this technique have been reported, and will need to be confirmed by independent groups (Barker, 2014).

In the following section, current knowledge of ovarian cancer stem cells will be discussed.
Evidence for Ovarian Cancer Stem Cells

Identification of a cancer stem cell involves a series of more or less defined experiments to test whether the certain stem-like features are present within a purified population of cancer cells. The sources of these cell populations can be cancer cell lines, tumors generated from mouse models, or preferably, primary ovarian cancer samples. In order to be classified as a cancer stem cell, the following criteria must be met (Table 5):

Table 5. Qualities of a Cancer Stem Cell. Adapted from (Shah & Landen, 2014)

- Increased tumorigenic capability in xenograft models
- Clonogenic (ability for form a colony from a single cell)
- Unlimited self-renewal
- Pluripotency (ability to produce distinct, differentiated daughter cells)
- Ability to recapitulate heterogeneous parental disease
- Chemoresistance
- Radiation resistance
- Form spheroids in suspension

The first paper to describe stem-like behavior in ovarian cancer emerged in 2005. Bapat and colleagues isolated and propagated ascitic cell spheroids from a patient with advanced stage disease, and characterized 10 representative clones that had spontaneously immortalized in culture. RT-PCR detected up-regulation of vimentin, E-Cadherin (in all but one clone), cytokeratin 18, c-met and EGFR, and surface adhesion molecule CD44. Stem cell pathway proteins Snail and Slug were present, although the pathway in which they are involved, c-kit-SCF, was
expressed differentially among the clones, suggesting they existed at different phases in the epithelial to mesenchymal spectrum. Only two of 10 representative clones were able to form colonies in soft agar, (clonogenic), and they were found to express stem cell mediators, Nestin, Nanog, and Oct4. These clones, when injected into nude mice subcutaneously and intraperitoneally both formed tumors and metastases, although one was more aggressive than the other, and had a more epithelial phenotype which resembled the patient sample. Each of the clones were capable of regenerating tumor upon serial implantation to other mice (Bapat, Mali, Koppikar, & Kurrey, 2005). This study met some but not all of the criteria listed in Table 5, namely the characterization of heterogeneity and pluripotency was not addressed, nor was chemoresistance. Regardless, this study marked the first evidence for the possibility of ovarian cancer stem cells, and a plethora of subsequent investigations followed.

Potential cancer stem cells are isolated from a heterogeneous population of primary tumor cells in various ways – this process is also called “prospective identification”. In the above example, Bapat and colleagues had selected clones based on behaviors in cell culture, such as clonogenicity in agar and ability to form spheroids, or anchorage-independent structures, that are characteristic of normal stem cells (Sukach & Ivanov, 2007). Other studies have followed this method of selecting clones in ovarian cancer (S. Zhang et al., 2008) and other cancers such as breast (Saadin & White, 2013). Another strategy for selecting subpopulations is based on cell surface markers that are known to be associated
with stem cells, such as CD133. Fluorescence-activated cell sorting (FACS) is the method of choice for this selection process, and many cell surface markers have been employed in the search for ovarian cancer stem cells (Table 6).

CD133 is of particular interest because of its unique expression in hematopoietic and epithelial stem cells, and since it had been used to successfully identify tumor initiating cells in brain, pancreatic, liver, skin, prostate and colon cancers (Baba et al., 2009). The function of CD133 is largely unknown. Several studies have shown that CD133+ ovarian cancer cells, isolated from patient samples as well as those originating from ovarian cancer cell lines, have enhanced tumorigenecity compared to CD133- cells (Baba et al., 2009; Curley et al., 2009; Kusumbe, Mali, & Bapat, 2009). Interestingly, one study showed that CD133+ cancer cells have a remarkable ability to induce angiogenesis, suggesting a potential role in modifying and creating heterogeneity in the extracellular environment (Kusumbe et al., 2009). Despite numerous studies that implicate CD133 as a stem cell marker, doubt remains as to the true relevance of CD133 in identifying a cancer stem cell, as it has been shown that CD133- cells can also be tumorigenic, and tumorigenicity of CD133+ cells seems to depend on the subtype of cancer and the context in which it is tested (Stewart et al., 2011).
Table 6. Ovarian Cancer Stem Cell Markers. Adapted from (M. M. Shah & Landen, 2014).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Conclusions</th>
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<tbody>
<tr>
<td>CD133+</td>
<td>Increased tumorigenesis and angiogenesis, associated with poor prognosis (Baba et al., 2009; Curley et al., 2009; Kusumbe et al., 2009; Silva et al., 2011; Jing Zhang et al., 2012)</td>
</tr>
<tr>
<td>CD44+/MyD88+</td>
<td>Increased tumorigenesis and chemoresistance (Alvero et al., 2009)</td>
</tr>
<tr>
<td>CD44+/CD117+</td>
<td>Increased tumorigenesis and chemoresistance (S. Zhang et al., 2008)</td>
</tr>
<tr>
<td>CD44+/CD24-</td>
<td>Spheroid formation, recapitulate parental tumor (cell lines) (Shi et al., 2010)</td>
</tr>
<tr>
<td>CD44+/CD24+</td>
<td>Increased tumorigenesis (primary tumor/xenograft) (Gao, Choi, Kang, Youn, &amp; Cho, 2010)</td>
</tr>
<tr>
<td>ALDH1A1+</td>
<td>Increased tumorigenesis and pluripotency (Landen et al., 2010)</td>
</tr>
<tr>
<td>ALDH1A1+/CD133+</td>
<td>Increased tumorigenesis, chemoresistance and self-renewal (Kryczek et al., 2012; Silva et al., 2011)</td>
</tr>
<tr>
<td>ABCG2 (side pop’n)</td>
<td>Increased tumorigenesis, chemoresistance and self-renewal (Dou et al., 2011; Hu, McArthur, &amp; Jaffe, 2010; Kobayashi et al., 2011; Szotek et al., 2008)</td>
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Much conflicting evidence has been put forth concerning the implications for cells expressing certain markers. Of particular controversy has been the cell marker aldehyde dehydrogenase (ALDH), a cytosolic enzyme which has been associated with a stem cell behaviors in a number of cancers including breast and colon (Huang et al., 2009; Kim et al., 2013). In some cases, ALDH expression is associated with poor clinical outcomes (Deng et al., 2010; Landen et al., 2010; Silva et al., 2011) while in others it is not (B. Chang et al., 2009; Penumatsa, Edassery, Barua, Bradaric, & Luborsky, 2010). An explanation for these dichotomous findings is that ovarian cancer is a heterogeneous disease –
what may be true in one case may not apply to another. Moreover, Chang et al found that ALDH had the highest expression in early-stage endometrial ovarian cancer, and correlated with a longer survival, however failed to distinguish between various subtypes of carcinoma that have inherently different prognoses.

Interestingly, one study may explain previous conflicting evidence for the role of CD133 as a CSC marker. This group found that tumorigenicity of cells expressing CD133 was variable and depended on co-expression with ALDH (Landen et al., 2010), suggesting that the CD133+ cells that were found to lack tumorigenic capacity in other studies may have been negative for ALDH. These findings illustrate the complex problem of prospective identification of cancer stem cells, and that one marker is likely insufficient for a confident definition. Moreover, even multiple markers that identify cancer stem cells in certain cases may not identify them in others, and the CSC phenotype likely depends on the cell of origin and unique tumorigenic process that occurred in a particular patient.

A useful perspective into the nature of stem cell markers was obtained in one study that used FACS to separate CD24+ and CD24- cells, originally isolated from the same primary ovarian cancer tumor, and subsequently compared them for expression of stem cells genes (Gao et al., 2010). RT-PCR revealed that CD24 expression is accompanied by enrichment of β-catenin, Bmi-1, Notch1 and Notch4, and lower levels of E-Cadherin (Figure 22). This result is interesting because it suggests a correlation between CD24 and cancer stem cell function,
and identifies candidate pathways that might be involved. Moreover, the use of a proper control – cells were derived from the same source, cultured in the same conditions, and separated based only upon their expression of CD24 or lack thereof – removes variability and doubt from the findings. More studies like this, which help to correlate programs associated with the surface markers and stem cell behaviors are needed – as personalized medicine envisions individual patient sample analysis for stem cell markers informing a targeted therapeutic approach. Further, understanding these relationships will bring confidence for the use of surface markers in identifying ovarian cancer stem cells and put to rest the confusion that has arisen from attempts to oversimplify the multifaceted characteristics of cancer stem cells by hastily assigning to them a handful of surface markers, many of whose functions remain unknown especially in relation to stem cell biology.
In summary, a multitude of reports have claimed that cancer stem cells are “defined” by the presence of certain markers, however there is obviously conflict about which are relevant. It seems there are a variety of markers that could be potentially used to identify cancer stem cells, depending on the source of the original population, suggesting that cancer stem cells themselves are heterogeneous. A more accurate statement, therefore, may be to claim that cancer stem cells may possess certain surface markers, such as CD133, but aren’t necessarily defined by them. Surely, before we can define cancer stem

**Figure 22.** RT-PCR reveals connection between CD24 and stem cell function. Cells isolated from a primary tumor of ovarian cancer were purified and separated by FACS based on the presence of CD24 surface marker. CD24 expression was accompanied by increased levels of β-catenin, Bmi-1, Notch1 and Notch4, as well as decreased expression of E-cadherin. Taken from (Gao et al., 2010).
cells based on these markers, we must first understand their functions and how they relate to, or regulate, stem cell behavior. In any case, it seems we are just beginning to crack the surface on what defines a cancer stem cell, and the variety of cell surface markers, pathways and mechanisms that define them.

In contrast to the work being done in prospective identification of cancer stem cells in vitro, clinical data is solidifying the notion that stem-cell like populations exist in ovarian cancer. The significance of these populations has been investigated in terms of chemoresistance, prognosis, and targeted therapy. A robust study by Steg et al examined 45 matched primary/recurrent tumor pairs of aggressive high grade serous ovarian carcinoma for the presence of stem cell markers and pathways. Specifically, immunohistochemistry and quantitative PCR revealed that ALDH1A1, CD44, and CD133, along with genes in the TGF-β, Hedgehog, Notch, and Wnt pathways were elevated in specimens collected immediately after treatment. Interestingly, upon tumor recurrence (12-18 months later), the levels of these markers went back to the original values in the primary tumor sample – suggesting the stem-like population which persisted during treatment differentiated to recapitulate the original disease heterogeneity within this timeframe (Steg et al., 2012). In another study, the extent of cancer stem cells (identified by cell surface marker CD44) that were found to be in biopsy samples was negatively predictive of progression-free survival in early stage ovarian cancer (Steffensen et al., 2011). Furthermore, a targeted therapeutic approach aimed at CD44+ used a novel nanoscale siRNA drug delivery system
showed this drug was able to irradiate patient-derived malignant tumors \textit{in vitro} and \textit{in vivo}, providing support that CSCs defined by CD44+ are viable targets for therapy (V. Shah et al., 2013).

Although many questions remain about the nature of cancer stem cells, abundant evidence has proven their existence in ovarian cancer, and clinical relevance in chemoresistance and as potential targets for future therapies. Further work is needed to characterize ovarian cancer stem cells and the variability of cell surface markers expressed.

\textit{Validity of the cancer stem cell model in predicting intratumoral heterogeneity}

Conceptually, there are various reasons why a stem cell model makes sense in explaining the pathogenesis of ovarian cancer. Shah and Landen present several ideas: the clinical course itself as evidence – the fact that certain cells respond differently to treatment and can survive chemotherapy – suggests that a rare resistant population exists that evades traditional targeting of rapidly proliferating cells. Further, there is evidence for stem cells in the fallopian epithelium and stem-like cells on the ovarian surface epithelium, although there is much to be understood about these cells and how they function, it is important evidence that stem cell precursors do exist in the tissues speculated to give rise to ovarian cancer. In fact, a recent publication revealed a stem cell niche in the ovarian surface epithelium that was particularly prone to malignant transformation after inactivation of tumor suppressor genes TP53 and Rb1 (Flesken-Nikitin et al.,
2013). Lastly, the fact that ovarian cancer presents with a wide range of phenotypes (serous, clear cells, mucinous, etc) and that these subtypes are often found mixed in the same patient, suggests the possibility of a common cell of origin – a stem cell with remarkable differentiating capabilities (Shah & Landen, 2014).

Validating the cancer stem cell model will require designing more robust protocols to identify and study these subpopulations of tumor initiating cells. Much criticism has been applied toward the cancer stem cell theory in general due to the unreliability of stem cell markers in predicting tumorigenic cell populations in large cohorts of different patients and cancer types (Magee, Piskounova, & Morrison, 2012; Meacham & Morrison, 2013). In fact, isolation of cancer stem cells based on cell surface markers could be a flawed approach to capture an elusive population of cells which are as heterogeneous as the disease itself, displaying a multitude of cell surface markers, many with unknown functions, which vary widely from patient to patient with the same disease and therefore aren’t necessarily universal identifiers. Vermeulen et al has taken a new approach toward identifying cancer stem cells not by cell surface marker, but based on the activity of signaling pathways which are known to be associated with stem cell functions (Vermeulen et al., 2010). Finding cancer stem cells based on function and behavior, rather than surface markers, avoids putting the cart before the horse, which has unfortunately led to much confusion and doubt for the cancer stem cell model.
Other critics point out the nature of the tumorigenesis assay that involves the xenograft transplantation of cancer stem cells into immunocompromised mice. They argue this experiment may be misleading due to the foreign microenvironment, which may not allow certain cell populations to grow that would otherwise be tumorigenic in the host (Magee et al., 2012; Meacham & Morrison, 2013). Further evidence of the importance of the microenvironment is provided by Abelson et al, which shows that cancer cell populations that wouldn’t grow in traditional xenograft models are enabled by providing a human embryonic stem cell (hESC)-derived cellular microenvironment. Moreover, the function of cancer stem cells is ‘niche-dependent,’ meaning self-renewing capacity of cancer stem cells is adaptive and depends on signals from the microenvironment; indeed, it is true that xenotransplantation has its limitations in studying cancer stem cells (Abelson, Shamai, Berger, Skorecki, & Tzukerman, 2013). Thus, it is generally agreed that the model for studying cancer stem cells, in particular for identifying which cells display tumorigenic potential, could benefit from a redesign.

Genetic/epigenetic variety is often thought of as being too vast to be explained by cancer stem cell differentiation alone. The argument is that genetic mutations are permanent changes in the genome, while stem cell differentiation is reversible, involving epigenetic, plastic changes that alter gene expression (Chen & Dent, 2014). These reversible changes in genome architecture cannot explain the permanent changes in the genome sequence brought about by mutations.
(Magee et al., 2012). On the other hand, one study used mathematical models to simulate cancer progression according to the stem cell theory, and have found if cancer did progress according to this theory, increased phenotypical heterogeneity would be seen in tumors, vs. a non-CSC model (Sottoriva et al., 2010). If one were to consider the relatively small number of genetic mutations, such as BRCA1/2, consistently found in ovarian cancers, in contrast to the much larger number of epigenetic changes involved, it seems likely that the cancer stem cell model, which generates heterogeneity through epigenetic changes, could be valid in explaining the majority of intratumoral heterogeneity found within ovarian cancer.

The heterogeneous nature of the microenvironment, which includes fibroblasts, immune cells and blood and lymph vessels, is known to be regulated by stem cell function (Briest et al., 2012). For example, cancer stem cells have been shown to induce angiogenesis in several reports (Kusumbe et al., 2009; Li & Zhang, 2013; Salnikov et al., 2013). Interactions between cancer stem cells and the surrounding stroma have been shown to promote metastatic processes and maintain the stem-like state (Malanchi et al., 2012); conversely, cancer associated fibroblasts (CAFs) have been shown to increase the “cancer stem cell pool” through bone morphogenetic protein 2 (McLean et al., 2011). The importance of the stem-cell niche environment has been demonstrated with regard to stem cell plasticity as well (Abelson et al., 2013). Whether or not cancer stem cells alone are responsible for intratumoral microenvironmental
heterogeneity remains a question. Non-stem cell phenotypes are shown to be capable of influencing the microenvironment, thus it is likely that a combination of both CSC and non-CSC cellular signaling is responsible for the dramatic rearrangements seen in the tumor microenvironment, with perhaps CSCs having an enhanced ability to orchestrate these changes (Oskarsson, Batlle, & Massagué, 2014).

The ability of cancer stem cells to undergo epithelial to mesenchymal transitions (EMTs) is an area of increasing interest in cancer research, as it has been implicated in metastatic processes, and is perhaps offers the strongest support for the cancer stem cell model. It has been shown in various cancers that malignant metastases share features of both EMT and stem cell traits (Thiery, Acloque, Huang, & Nieto, 2009). Indeed, many of the same pathways that control EMT also regulate stem cell maintenance, self-renewal and differentiation, such as Wnt and Notch (Yang & Weinberg, 2008), both of which are found to be dysregulated in ovarian cancer (Arend et al., 2013; Cancer Genome Atlas Research Network, 2011; Mak et al., 2012). The epigenetic gene silencer, ‘enhancer of zeste homolog 2’ (EZH2) causes the silencing of E-cadherin expression, a critical step in the EMT process (Onder et al., 2008); at the same time, EZH2 has been investigated as a stem cell marker in ovarian cancer, as it appears to be overexpressed in populations of chemoresistant cancer cells (Rizzo et al., 2011). There are numerous examples of the overlapping nature of
the cancer stem cell phenotype with EMT programs; the evidence linking these
two phenotypes is abundant.
Still, the question ‘which came first’ is a matter of intense and important debate
(Chang & Mani, 2013). It is possible that normal stem cells undergo a
transformation to become cancer stem cells, and subsequently undergo EMT to
metastasize; yet another possibility is that mature, epithelial cancer cells de-
differentiated to a more mesenchymal phenotype, thereby acquiring stem-like
traits secondarily to initiation of an EMT program. A potential mechanism for the
latter was proposed – evidence suggests that cancer associated fibroblasts are
capable of initiating an EMT program in tumor cells through cytokine signaling,
and that these tumor cells concurrently develop “stemness,” such as
tumorigenetic capability and self-renewal (Giannoni et al., 2010). This does not
prove, however, that this is the sequence of events for any or all cancer types;
the many complex interactions between stem cells and the surrounding stroma
adds profound uncertainty to this proposed mechanism. Answering the question,
‘which came first,’ will require a deeper understanding of the cell of origin for
different cancers through new experimental techniques such as lineage tracing
(Alcolea & Jones, 2013; Blanpain & Simons, 2013). Until these new techniques
are mastered and applied to malignant solid tumors, the validity of the cancer
stem cell model in predicting heterogeneity required for metastasis remains
plausible but unproven.
CONCLUSIONS AND FUTURE DIRECTIONS

Intratumoral heterogeneity (ITH) has implications for metastases, drug resistance and recurrence. A deeper understanding of the nature of ITH will allow for more effective treatments. Each source of heterogeneity – genetic, epigenetic, microenvironmental, and differentiation/cell plasticity – has proven influential in various cancer types. Explaining how this heterogeneity arises in different cancers may involve a combination of both clonal evolution and cancer stem cell models, but to varying degrees depending on the cancer type. For example, melanoma does not fit as well within the CSC model as compared to other cancers, and its progression, metastasis, and resistance to treatment may be explained best by a model which emphasizes genetic instability and/or cooperativity between subpopulations.

Cell plasticity has been recently suggested to be a critical component of tumor progression and metastasis, and the processes that control cell differentiation and de-differentiation are prominent in cancer stem cells. Thus, the importance of reversible epithelial-mesenchymal phenotypes in cancer stem cells has emerged, and shifted the theory from a rigid hierarchical model to a dynamic one, wherein differentiated cells can revert back to their stem-like state. This new model will permit a wider array of cancer types to be described within its context.
Whether or not the evolution of ovarian cancer fits the cancer stem cell model remains controversial, however, data suggests that a hierarchy of tumorigenic and non-tumorigenic cells does exist in ovarian cancer. A big question is whether these tumorigenic cells start out as normal stem cells that undergo cancerous transformations, or whether their stem-like properties are gained through genetic alterations. Knowing the answer to this question will have important implications for treatments that target stem cell phenotypes. In any case, one cannot deny that small, rare subpopulations of cancer cells have been found to exhibit significantly more potent tumor initiating activity than the majority of tumor cells found in ovarian cancer. If cancer stem cells are involved in metastases and recurrence, as evidence suggests, it will be important to continue to learn more about how to purify and identify these cells for targeted therapy.

Future work in the field of cancer stem cells should include more systematic characterization of the various types of cancer stem cells found in ovarian cancer patients, as well as development of robust protocols to study their behavior both in vitro and in mouse models. Understanding the diversity of CSCs found within ovarian cancer will be critical for future personalized medicine approaches that employ targeted therapies to eradicate the tumorigenic populations, coupled with standard chemotherapy to eliminate the rapidly dividing bulk of tumor. Furthermore, exploring the possibility of a stem cell origin for ovarian tumors should employ lineage tracing experiments, similar to what has been done for
intestinal adenomas (Alcolea & Jones, 2013). Lineage tracing allows for direct observation of cell proliferation in vivo using fluorescent reporters that are passed down from parent to daughter cell. One group has used this technique to study the cancer-prone stem cell niche at the ovarian hilum, (Flesken-Nikitin et al., 2013), however longitudinal studies that show the nature of progression toward metastatic disease are needed to further characterize the stem cell contribution during this transformation.

As we understand more about the causes of intratumoral heterogeneity, we will be more informed to design drugs that fight against the advantages, which this heterogeneity most certainly lends to the tumor progression. Further exploration and characterization of the role of cancer stem cells in establishing intratumoral heterogeneity, and how it contributes to tumor progression, is clearly needed.
APPENDIX I

The most common subtypes of ovarian cancer are outlined below (information taken from (Robert J Kurman & Shih, 2010; McCluggage, 2011; Prat, 2012).

<table>
<thead>
<tr>
<th><strong>High Grade Serous Carcinoma (HGSC)</strong></th>
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<tr>
<td>• Account for 68-71% of ovarian cancers</td>
</tr>
<tr>
<td>• Aggressive phenotype, typically present at advanced stage, with poor prognosis</td>
</tr>
<tr>
<td>• Histological appearance is slightly glandular, with a slit-like lumen, intermediate cell size, with occasional giant cells having large nucleoli (Figure 4a). Cells are ciliated columnar and produce a clear fluid.</td>
</tr>
<tr>
<td>• The only ovarian subtype with a known risk factor of hereditary BRCA mutations.</td>
</tr>
<tr>
<td>• Commonly express p53, BRCA1, WT1 and p16, and high proliferation is indicated by nuclear expression of Ki-67. Also may express estrogen receptor (ER).</td>
</tr>
<tr>
<td>• Cell of origin is a matter of debate as being either from the fallopian tube of ovarian surface epithelium.</td>
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<tr>
<td>• Typically sensitive to first round chemotherapy of platinum/taxane, however recurrent, resistant disease will develop and ultimately lead to death of the patient.</td>
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<th><strong>Low Grade Serous Carcinoma (LGSC)</strong></th>
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<tr>
<td>• Uncommon, accounting for &lt;5% of ovarian cancers.</td>
</tr>
<tr>
<td>• Relatively benign phenotype, however should be monitored closely as it can transform into HGSC.</td>
</tr>
<tr>
<td>• Forms small papillae with psammoma bodies in the stroma (Figure 4b). Uniformity of nuclei is the key distinguishing feature in comparison to HGSC microscopically.</td>
</tr>
<tr>
<td>• Similar expression profiles to HGSC, although Ki-67 is far less, indicating reduced proliferation reflective of its generally benign behavior.</td>
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<tr>
<td>• Although responsiveness to treatment has not been determined, preliminary data suggest insensitivity to standard chemotherapeutics.</td>
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<th><strong>Mucinous Carcinoma (MC)</strong></th>
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<tr>
<td>• Accounts for 3-4% of ovarian cancers.</td>
</tr>
<tr>
<td>• Mostly benign (80%) phenotype, however invasion can occur and be difficult to detect.</td>
</tr>
<tr>
<td>• Can resemble gastrointestinal metastases, because the cells are non-ciliated columnar, and produce a viscous fluid similar to cells of the GI tract (Figure 4c). Malignant mucinous carcinomas are heterogeneous and may contain benign components as well, which may confound diagnosis if proper sampling size is not taken.</td>
</tr>
<tr>
<td>• Cells express CDX2 and KRAS which are also found in gastrointestinal cells. Cytokeratin 7 (CK7) is often expressed and can be used to distinguish from colorectal metastases. In contrast to endometrioid and serous tumors, MCs are negative for estrogen receptor and WT1.</td>
</tr>
<tr>
<td>• Cell of origin is unknown.</td>
</tr>
<tr>
<td>• Response to treatment is largely unknown. Most MCs are benign, and malignant MCs are difficult to diagnose due to their heterogeneity. Adding to the rarity of the disease, this makes research into its chemosensitivity a formidable challenge.</td>
</tr>
</tbody>
</table>
**Endometrioid Carcinoma (EC)**
- Accounts for 10% of ovarian cancers.
- Very benign phenotype and often detected early, therefore having the most favorable prognosis of all subtypes.
- Histologically similar to the appearance of uterine cell lining, and usually EC is associated with parallel disease of the endometrium – either carcinoma, cysts, or endometriosis. In 50% of cases, squamous differentiation can occur (Figure 4d). Although EC can be classified as low grade or high grade, most fall into the low grade category, while high grade is almost indistinguishable from HGSC.
- Only subtype with known risk factor of hereditary non-polyposis colon cancer (HNPCC). Additional risk factor is atypical endometriosis, especially with PTEN deletion, which is also associated with the clear cell subtype.
- Mutations in AT-rich interactive domain 1A gene (ARID1A) are present in EC, and have been found in associated endometriotic tissue. This gene is part of the SWI-SNF-A complex that effects transcription, essentially behaving as a tumor suppressor gene via its encoded protein, BAF250. This mutation is also present in clear cell carcinoma. Beta-catenin over-expression is often seen in ovarian EC as a consequence of CTNNB1 gene mutations. Mutations in PTEN cause activation of the PI3K-AKT pathway that inhibits apoptosis, but this pathway can also be activated through mutations in PIK3CA gene which encodes the p110 catalytic subunit of PI3K. ECs express vimentin, cytokeratins, epithelial surface antigen, and estrogen and progesterone receptors, but are negative for WT1.
- Cell of origin is proposed to arise from the endometrium or myometrium.

**Clear Cell Carcinoma (CCC)**
- Account for 10% of ovarian cancers.
- Many present at early stages with favorable prognosis, however advanced stage disease has the worst prognosis of all ovarian cancer subtypes.
- Cells have a clear cytoplasm with presence of hobnail phenotype – bulbous protruding shape extending from the surface (Figure 4e). Also contain multiple papillae, dense basement membrane and hyaline bodies in a quarter of cases.
- Risk factors include disease of the uterus, similar to endometrioid carcinoma.
- Unlike HGSCs, CCC is negative for BRCA, ER and WT1, while they are usually positive for HNF1-beta, and ARID1A mutations in about half of all cases. Similar to EC, ARID1A mutations and absence of BAF250 protein are often found in adjacent endometriotic tissue. In particular, overexpression of the transcription factor HNF1-beta in CCC has a large impact of pathogenesis because of its regulatory role of several genes including dipeptidyl peptidase IV, osteopontin, angiotensin converting enzyme 2, annexin 4 and UGT1A1, which are respectively involved in glycogen synthesis, progesterone-regulated endometrial secretory protein, ferritin induction/iron deposition/antiapoptosis, paclitaxel resistance, and detoxification. It is believed that CCC progression is hindered due to high expression of Rho-GDP dissociation inhibitor gamma (ARHGDIG) mRNA, which inhibits RHO GTPase pathways involved in cytoskeletal regulation and tumor progression.
- Cell of origin is proposed to arise from endometrium or myometrium.
- CCC is typically unresponsive to chemotherapy because of low replication rates and relatively stable genetics.
APPENDIX II
Table 4. Regulators of Embryonic Stem Cell Differentiation. Adapted from (Heng & Ng, 2010; Yamanaka & Ralston, 2010) '  

<table>
<thead>
<tr>
<th>Regulatory Factor</th>
<th>Role</th>
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<tbody>
<tr>
<td>Yap/Taz</td>
<td>Through phosphorylation by Lats1/2 of the Hippo pathway, and mechanisms involving cell-cell contact, Yap/Taz localize to nucleus of cells destined to become trophoblast lineage, and remain in cytoplasm of cells destined to differentiate into inner cell mass.</td>
</tr>
<tr>
<td>Tead4</td>
<td>Binds to Yap/Taz in nucleus to activate DNA transcription of CDX2, required for development of trophoblast.</td>
</tr>
<tr>
<td>CDX2</td>
<td>Required for maturation and maintenance of trophoblast, suppresses formation of inner cell mass maturation by antagonizing Oct4.</td>
</tr>
<tr>
<td>Oct4</td>
<td>Required for inner cell mass formation, suppresses trophoblast formation by antagonizing CDX2. Maintains pluripotency when expressed at a neutral level, but if down or up regulated it will induce differentiation. Expressed in epiblasts and primordial germ cells in addition to inner cell mass.</td>
</tr>
<tr>
<td>Gata 4 and 6</td>
<td>Transcription factors for further differentiation of randomized cells within the inner cell mass into the yolk sac, which provides nutrients and polarity for the developing fetus.</td>
</tr>
<tr>
<td>Nanog</td>
<td>Specifies randomized cells within the inner cell mass which are destined to develop into the fetus or yolk sac. Dimerizes and maintains pluripotency, but low levels permit differentiation under influence of FGF-signaling. Forms complex with Smad1 to inhibit BMP-mediated differentiation. Works in parallel to Stat3 by binding members of the NF-kB pathway and inhibiting differentiation.</td>
</tr>
<tr>
<td>FGF, FGF4</td>
<td>Necessary for yolk sac development. FGF4 required for differentiation of epiblast layer.</td>
</tr>
<tr>
<td>Sox2</td>
<td>A pluripotent gene expressed along with Oct4 in epiblast stem cells. Maintains pluripotency and self-renewal. Also expressed in neural progenitor cells. Forms dimers with Oct4 to regulate a host of embryonic stem cell genes. Other homologs (Sox4, Sox11, Sox15) have similar capabilities.</td>
</tr>
<tr>
<td>KLF (2, 4, 5)</td>
<td>Zinc finger transcription factor that maintains pluripotency and self-renewal. KLF4 can cause reversion of epiblast cells into their precursor stem cells.</td>
</tr>
<tr>
<td>Ronin</td>
<td>Transcription factor that interacts with other proteins such as HCF-1 to form complexes that mediate pluripotency by suppressing genes for differentiation.</td>
</tr>
<tr>
<td>Tbx3, Esrrb</td>
<td>Transcription factors involved in preserving self-renewal.</td>
</tr>
<tr>
<td>Sal1, Rif1, Dax1, Hdac1, Nac1, Zfp281</td>
<td>Protein partners of Nanog.</td>
</tr>
<tr>
<td>c-Myc, n-Myc, Zfx, E2F1</td>
<td>Cluster of transcription factors that work synergistically with Nanog-Sox2-Oct4 cluster to effect transcription of genes necessary for self-renewal and pluripotency.</td>
</tr>
<tr>
<td>P300</td>
<td>Co-activator that binds to enhancer region associated with Nanog-Sox2-Oct4 cluster and may be involved in DNA looping for faster transcription.</td>
</tr>
</tbody>
</table>
REFERENCES


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