UNIVERSITY OF CALIFORNIA

Los Angeles

The Differential Expression of OCT4 Variants
in Head and Neck Squamous Cell Carcinoma

A thesis submitted in partial satisfaction
of the requirements for the degree of Master of Science
in Oral Biology

by

Ho-Hyun Sun

2012
The Differential Expression of OCT4 Variants in Head and Neck Squamous Cell Carcinoma

by

Ho-Hyun Sun

Master of Science in Oral Biology
University of California, Los Angeles, 2012
Professor Yong Kim, Co-chair
Professor Robert H Chiu, Co-chair

OCT4 is a factor responsible for maintaining stemness. When expressed in embryonic stem cells, OCT4 induces self-renewal and pluripotency. However, OCT4 expression has also been linked to cancer progression in a variety of tumors. To determine which isoform is responsible for the modulation of head and neck cancer progression, the expression levels of OCT4A and OCT4B were measured. We found that while normal keratinocytes do not express OCT4, stem cells express OCT4A and cancer cells express OCT4B. Furthermore, the overexpression of OCT4A in a head and neck cancer cell line led to the activation of traits (such as increased proliferation) associated with poorer prognosis. We conclude that OCT4A may be the isoform responsible for the progression of head and neck cancer, and that it may serve as a marker of “cancer stem cells” thought to be the driving force behind metastasis and recurrence.
The thesis of Ho-Hyun Sun is approved

Reuben Han-Kyu Kim

Shen Hu

Yong Kim, Committee Co-chair

Robert H Chiu, Committee Co-chair

University of California, Los Angeles

2012
DEDICATIONS

My work is dedicated to my mother, who fought two recurrences of cancer even as I worked to unravel its mechanisms. As I shifted between the laboratory and the hospital each day, it was her courage, strength, and support that gave me the determination to carry on.
# TABLE OF CONTENTS

Background

Pg. 1

Introduction

Pg. 4

Methods and Results

Pg. 16

Conclusions

Pg. 25

Discussions and Implications

Pg. 28

Figures and Diagrams

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The Human Oct4 Gene</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>Oct4A Coding Plasmid</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>OCT4 Isoforms in hESC</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>OCT4 Isoforms in Non-cancer Oral Keratinocytes</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>OCT4 Isoforms in HNSCC</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>The Quantitative PCR of Oct4 Isoform Expression</td>
<td>36</td>
</tr>
<tr>
<td>7</td>
<td>OCT4 Isoforms are missing in normal Tissue</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>OCT4 Isoform Expression in Cancer Tissue</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>HE Stain Images of Cancer Tissue</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>Verification of Oct4A Expression</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>Numbers of Transfected UMSCC2 Cells</td>
<td>41</td>
</tr>
<tr>
<td>12</td>
<td>UMSCC2 Growth Over 4 Days</td>
<td>42</td>
</tr>
<tr>
<td>13</td>
<td>Cadherin Expressions in HNSCC</td>
<td>43</td>
</tr>
<tr>
<td>14</td>
<td>UMSCC2 Cell Survival after 13uM Cisplatin Treatment</td>
<td>44</td>
</tr>
</tbody>
</table>

References

Pg. 45
BACKGROUND

Head and Neck Cancer As a Public Health Issue: Risks, Survival Rate, and Epidemiology

Head and neck cancer refers to a group of phenotypically similar cancers of the mouth, nasal cavity, pharynx, larynx, and the lips. A large majority of head and neck cancers are of epithelial origin, and are classified as squamous cell carcinomas\(^1\). The most common sub groups of head and neck squamous cell carcinoma (HNSCC) are oral and laryngeal squamous cell carcinoma, with over 100,000 cases arising in the U.S. between 1985 and 1994\(^2\). Common risk factors behind head and neck cancer include alcohol and tobacco (especially when used together)\(^3\), though infection by the human papillomavirus may cause over 25\% of all HNSCC\(^4\). Unfortunately, many cases are detected at later stages of disease\(^3\) and are often found with metastatic lymph node nodules\(^1\). Despite the advent of innovative therapies like antibody therapy, prognosis remains especially poor once the tumors have metastasized\(^5\).

HNSCC is a significant problem in the field of oncology. With a high recurrence rate and a lower than 50\% probability of five year survival\(^6\), HNSCC is the most frequent killer of young men in regions like Southern China\(^7\). It is the third most prevalent cancer in developing nations overall and the sixth most common worldwide\(^8\). Radiation therapy may be used, though surgery remains the preferred method of treatment for lower stage cancers (with relatively high rates of success). However, chemotherapy is the only viable option for patients with multiple metastases. Common chemotherapeutic agents include doxorubicin and cisplatin\(^3\).
The New Cancer Paradigm

Cancer is a disease of proliferation. It is the uncontrolled growth of mutated cells and the cells’ subsequent invasion into vital tissue\textsuperscript{9}. Unlike benign cells which die and grow as signaled, cancer cells spontaneously undergo mitosis while remaining unresponsive to apoptotic signals.

In the traditional view, cancer cells are categorized by two characteristics. First, they are thought to arise from somatic cells that have undergone at least two separate mutations: a “loss of function” mutation that downregulates the activity of a growth-suppressive gene, and a “gain of function” mutation that boosts the expression and/or effectiveness of a proliferative gene\textsuperscript{9}. Second, cancer cells are also thought to actively change their genomic expression, acquiring new morphology and epigenetic modifications as the traits of the daughter cells deviate further from those of their progenitor\textsuperscript{10,11}. However, this generalized view has been largely reorganized with the advent of stem cell biology, as researchers find more and more parallels between cancer cells and stem cells.

The purpose of this research is to ascertain the dual role of the key stem cell molecule OCT4 in stem cells and head and neck cancer, and to better understand exactly what carcinogenesis is. As a master embryonic transcriptional switch, OCT4 is perhaps the most important molecule in modulating stem cell differentiation as well as the growth and proliferation of cancer. Its contribution to cancer progression is well documented in a wide variety of tumors, including those of the head and neck. Furthermore, it would be a wise approach to distinguish the differences in OCT4 isoforms and to separately categorize their individual contribution to carcinogenesis. The relatively new distinction of the OCT4A and OCT4B isoforms means that their individual mechanisms have not yet been detailed.
By outlining the common molecules and pathways between stem cells and HNSCC, we may provide additional insight into the cancer mechanism that could contribute to the formulation of more effective future therapies. If we can correctly describe cancer in terms of stem cells, we may succeed in using the signaling and regulatory mechanisms that control stem cell growth to curb cancer progression and maintain a healthy state.
INTRODUCTION

Stem Cells, Cancer, and the Stem Theory of Cancer

Today, stem cells are often hailed as the potential source of cures for every ailment imaginable. The stem cell's ability to form any cell type has established it as the go-to marketing mascot for healthcare corporations worldwide. In addition, leading researchers have for years attempted to direct stem cells’ growth and differentiation in order to create viable cultures of tissues or organs that can replace a sick one. Many hope that stem cells can be used to meet the shortages in many of the transplantable organs. But few realize the variations of stem cells that exist, and the capacity of stem cells to cause serious illnesses if not maintained under extremely intricate conditions.

A Variety of Embryonic and Adult Stem Cells Exist

Embryonic stem cells (ESCs) are pluripotent cells that are often isolated from the inner mass of the blastocyst. These are the stem cells most frequently mentioned in popular literature and the best understood in scientific circles. ESCs are characterized by their ability to proliferate quickly and indefinitely, and by their potential to differentiate into all three layers of the embryo (the ectoderm, the mesoderm, and the endoderm). These cells may be able to offer the widest variety of treatments in a clinical setting since they can differentiate into any tissue or organ.

On the other hand, adult stem cells (ASCs) are the multipotent/progenitor cells that can each form a handful of predefined cell types. Though putative ASCs also seem to exhibit indefinite life spans and rapid proliferation, they are not as well understood because of their rarity and the lack of definite methods of isolation. Still, ASCs may also offer valuable...
therapeutic potential when used to regenerate tissues specific to their niche. They may also be more reasonable to obtain as their isolation does not entail the risk, nor controversy of extracting cells from a fragile embryo. As of today, commonly isolated adult stem cell types include hematopoietic stem cells (HSCs), neural stem cells (NSCs)\textsuperscript{17}, dental pulp stem cells (DPSCs)\textsuperscript{18}, and Intestinal Stem Cells (ISCs)\textsuperscript{19}.

Both embryonic and adult stem cells share many of the genetic similarities that help maintain immortality. Cells like NSCs and ESCs share over 50\% overlap in genetic expression while many of the commonly isolated stem cell types express more than 200 of the same genes\textsuperscript{20} including the potency factor OCT4\textsuperscript{21}.

**Stem Cells and the “Conditional Cancer”**

The recent parallelization of cancer cells and stem cells has sparked controversy, though their mitotic and morphological similarities are undeniable. Like cancer cells, ESCs maintain extremely high rates of growth. The ESC cell cycle lacks the common G1 to S cell cycle check point\textsuperscript{22}, which many believe is analogous to the loss of function mutations that contribute to carcinogenesis. Also like cancer cells, ESCs undergo mitosis in the absence of proliferative signals from surrounding tissue. This is because these cells have the intrinsic need to quickly grow and produce large numbers of cells so that an entire organism can be produced within the matter of a few months. In turn, this growth allows for the rapid derivation of stem cell lines and the infinite proliferation of ESCs as well as cancer cells in culture\textsuperscript{23}.

In addition, both ESCs and cancer cells are differentiation-capable, as both are known to create large masses of heterogeneous tissue\textsuperscript{13,24}. In fact, the differentiation of ESCs themselves may be cancer-initiating, as xenografts of ESCs into immune-compromised individuals are
usually followed by aberrant differentiation and eventually leads tumors called teratomas\textsuperscript{25}. Therefore, it may not be unreasonable to categorize stem cells as “conditional cancer cells.” The only difference between a malignant cancer cell and a supposedly benign stem cell is that the stem cell’s extreme proliferative potential is (usually) directed and kept under check when left to grow in the womb.

**The Stem Cell Theory of Cancer**

The obvious similarities between stem cells and cancer cells have led to the “stem cell theory” of cancer. There are indications that many of the carcinogenic genes are in fact genes intended for maintaining the self-renewal and growth potentials of ESCs. The ESC genes are switched off during differentiation, but mutations may cause their deregulation to initiate cancer.\textsuperscript{26}.

Unlike the traditional view, the stem cell theory states that carcinogenesis is the reversion of an adult cell into an uncontrolled but ESC-like state. The accumulation of a sufficient number of the relevant mutations can induce ESC-like growth and differentiation. This may then create a “cancer stem cell” (CSC) that proliferates and differentiates to imitate a grotesque version of embryonic organogenesis\textsuperscript{26}. The result is a pseudo-functional “organ” (positioned at an inappropriate physiological locale) that not only disrupts the function of nearby tissue but can outgrow its surroundings to spill over into new locations. Meanwhile, some of the CSCs would remain within the organ-like mass to continue dividing to maintain the pseudo-organ’s size, similar to a hematopoietic stem cell maintaining a bone marrow\textsuperscript{27}.

The controversy in this model lies in the source of the cancer stem cell. Some sources indicate that adult stem cells are the predecessors to cancer stem cells\textsuperscript{28}. In this model, the pre-
cancer cell is an adult stem cell that already expresses some of the proliferative and differentiative capabilities of an ESC. Theoretically, these ASCs would require the activation of only a few more ESC genes to become a CSC. In fact, the knockdown of a single gene in mesenchymal stem cells has led to the formation of leiomyosarcoma-like smooth muscle tumors in mice\textsuperscript{29}. The “adult stem cell model” gains additional credence because the long life spans of ASCs give them greater time and probability of accumulating mutations.

On the other hand, others show that the conversion of non-stem adult cells into an ESC-or CSC-like state is possible. There is evidence that manipulation of four or less genes can create induced pluripotent stem cells (iPSCs) within a variety of non-stem adult cells\textsuperscript{30,31}, though the creation of iPSCs from adult stem cells seems to require even fewer manipulations\textsuperscript{32}. There is also indication that mutation of key genes like Beta-catenin in mouse adult somatic cells may activate several downstream stem cell-related genes to cause cancers\textsuperscript{27}. Terminally differentiated adult cells are much more common than their stem cell counterparts, and the “reversion to a stem cell model” may better support the widespread nature of cancers worldwide.

Regardless of the actual source of CSCs, both models agree that putative CSCs of many different cell types maintain similar characteristics such as chemoresistance\textsuperscript{33–36}. Both models also suggest that the activation of ESC factors may play a vital role in carcinogenesis and cancer progression. And as one of the factors used to create iPSCs from both neural stem cells and somatic cells\textsuperscript{30,32}, OCT4 may play a significant role in CSC biology.

**OCT4 is a Crucial Stem Cell Factor**

OCT4 is a crucial molecule in both embryonic and adult stem cells. OCT4 (also known as Octamer 4, OCT3, OCT3/4, POU Domain Class 5 trascription factor 1, or POU5F1) is an
embryonic stem cell transcription factor implicated in the maintenance of potency and self-renewal\textsuperscript{37}. It is a vital suppressor of ESC differentiation during the early stages of embryogenesis that prevents premature lineage commitment. OCT4 is also classified as a homeodomain transcription factor belonging to the POU group of proteins. It has recently garnered much interest because it was used to induce pluripotency in somatic cells\textsuperscript{30,32,38} and to cause tumor-like dysplasia in mice through dedifferentiation of epithelial cells\textsuperscript{39}. Previously, it was known that OCT4 is essential to maintaining stem cell self-renewal and that its downregulation led to improper timing of differentiation\textsuperscript{40,41}. OCT4 has also been implicated in the maintenance of adult stem cells\textsuperscript{21}, and may serve as markers of cancer stem cells in a variety of cancers\textsuperscript{42,43}.

\textbf{The Different Varieties of the OCT4 Isoforms}

There are two known protein isoforms (OCT4A and OCT4B) and three known transcripts (Oct4A, Oct4B and Oct4B1) of OCT4\textsuperscript{44}. A distinct “OCT4B1 protein” has not yet be characterized\textsuperscript{45}, perhaps because the Oct4B1 mRNA contains two separate stop codons: one in its third exon and another in its sixth and most downstream exon\textsuperscript{44}. The transcription of Oct4B1 cDNA tagged with HA have yielded protein bands around 20 kDa\textsuperscript{46}, though over-expression assays of the cDNA suggest that the Oct4B1 transcript may simply be spliced to form the Oct4B mRNA\textsuperscript{47}. As a result, the determination of Oct4B1 gene expression so far has relied solely on reverse-transcriptase PCR. Because of the uncertainties surrounding the existence of the OCT4B1 protein, our research has focused on the two better established variants of OCT4.
**OCT4A May Induce Cancer Stemness**

Though many publications fail to distinguish among the different variants of OCT4, it is widely believed that the focus of most investigations until now has been the OCT4A variant. OCT4A is the 45kDa isoform whose role as the inducer of stemness traits such as self-renewal and potency is well known\(^48\). It is likely the purported Yamanaka Factor isoform used to induce pluripotency in adult somatic cells, as the OCT4 antibody used by Yamanaka et. al does not cross-react with the OCT4B isoform\(^30\). OCT4A is also the likely marker of cell stemness\(^49,50\). It is found in high levels in several different types of stem cells (both embryonic and adult)\(^51,52\) and also in stem cell cancers like embryonic carcinoma\(^37,53\), while our understanding of its roles in somatic cell cancers is limited\(^52,54\).

There are indications that OCT4A is expressed in a small subpopulations or “side groups” of cancer cell lines. In the case of prostate adenocarcinoma, one group has successfully isolated a small community of OCT4A expressing cells and found that their OCT4A levels correlated positively with the parent tumors' Gleason scores\(^55,56\). Several other investigators have also linked the expression of OCT4A (either explicitly through the intentional use of an OCT4A specific antibody/rtPCR primer, or implicitly by looking at the 45 kDa variant protein) to the expression of other cancer stem cell-related characteristics such as chemoresistance\(^55,57,58\). OCT4A expression was correlated to increased taxane resistance in prostate adenocarcinoma even though other important stemness factors such as KLF4, SOX2, and NANOG were not expressed\(^55\). Within lung cancer cells, OCT4A overexpressing cell lines showed greater expression of CD133 putative cancer stem cell marker as well as enhanced muscle layer invasion when xenografted in SCID mice\(^57\). Other traits enhanced by OCT4A overexpression include increased mobility and anchorage independent growth. Still, studies have confirmed the
elusiveness of OCT4A in the majority cancer cell lines\textsuperscript{59}, further mystifying its exact role in a non-stem niche.

**OCT4B is Generally Expressed in Non-Stem Cell Cancers**

Unlike OCT4A, the functional niche of OCT4B has not been studied extensively. OCT4B is the 35kDa isoform of OCT4 that was found to be missing in ESCs\textsuperscript{59}. There are indications that OCT4B may antagonize cell death\textsuperscript{59,60}, though it is not normally expressed in non-cancerous cells\textsuperscript{52} including embryonic stem cells\textsuperscript{59}. However, since OCT4B cannot activate the downstream targets of OCT4A\textsuperscript{48}, the two isoforms should not simply represent differentially effective versions of the same transcription factor. This notion is strengthened by exclusivity: the two isoforms are seldom expressed together in a single cell type. OCT4B is generally detected in non-stem cancer cells that do not express OCT4A\textsuperscript{61}, while OCT4A is mostly present in stem cells that do not express OCT4B\textsuperscript{54,59}.

**Structural Comparisons between OCT4A and OCT4B**

The structures of the OCT4 isoform proteins and transcripts are more alike than their functions. Both Oct4A and Oct4B mRNA are made up of five exons, of which the four downstream exons are congruent (Figure 1). This results in polypeptide forms that share the same C-terminus but are different at their N-termini\textsuperscript{62}. Only the OCT4A variant contains the 138 amino acid long N-transactivation domain while OCT4B contains the shorter 43 aa N-terminus domain. Both isoforms contain the same mid-sequence POU domain that is responsible for DNA binding\textsuperscript{62}. The two isoforms also share the putative nuclear localization signal (RKRKR), which may indicate that they traverse between the nuclear and cytoplasmic locales.
HNSCC and CSC

In general, traits like increased growth, resistance to therapy, invasion, and poor disease prognosis are associated with cancer stem cells \(^{28}\). In HNSCC, such cells that also express the CD44 mesenchymal stem cell marker have been identified \(^{63}\). As expected of CSCs, these cells composed a rare subpopulation (<10%) of the HNSCC tumor and were found to be differentiation-capable. Upon closer analysis, the cells were also found to express the self-renewal gene Bmi1 and to lack the differentiation marker Involucrin \(^{63}\). In their quest to find stem-like cells within head and neck cancer, Qunzho, et al have found like populations that express CD133, another adult stem cell marker \(^{35}\). Like the CD44 expressing cells of HNSCC, these cells were resistant to cytotoxic drug treatment as expected of cancer stem cells \(^{33,34,42}\) and possibly of adult stem cells as well \(^{64,65}\). They have also showed evidence that CD133 expressing cells highly express the embryonic stem cell factor OCT4, though it is not clear which OCT4 isoform that was detected.

OCT4 in HNSCC

The roles of the OCT4 isoforms in head and neck cancer remain obscure, though their expression patterns are expected to be analogous to those of other carcinomas and adenocarcinomas. The role of the OCT4A isoform is especially intriguing; it is likely the stemness molecule \(^{48}\) and its presence give us a better comparison between HNSCC cells and stem cells \(^{51}\). Several studies have been done to correlate the expression of overall OCT4 to cell phenotype.

In one study by Tsai et al, OCT4 expression in oral cancer was positively correlated with cisplatin resistance \(^{66}\). The investigators found that the level of OCT4 expression also correlated
with invasion, CD133 expression, and proliferation. Their study gives clues as to the importance of stemness molecules like OCT4 in head and neck CSCs, though the lack of distinction between the isoforms creates uncertainty.

A second study found that oral squamous cell carcinoma (OSCC – a division of HNSCC) patients with higher levels of OCT4 suffered from higher grades of cancer\textsuperscript{67}. In general, grading scales (such as the Gleason Score) correlate the aggressiveness and poor prognosis of disease with the cancer cells’ degree of dedifferentiation\textsuperscript{68}. As a consequence, the study correlated OCT4 expression to a dedifferentiated and thus a more stem-like phenotype. The study also indicated that the OCT4-positive HNSCC cells exhibit invasiveness and CD133 expression, and it is likely that the cells studied in this context were the same populations isolated by Tsai et al. Not surprisingly, a follow-up study concluded that OCT4A is the variant that was upregulated in another similar group of ASC-marker expressing HNSCC cells\textsuperscript{69}.

Though the previous investigations fall short of establishing the role of OCT4A in HNSCC, we expect that OCT4A is responsible for triggering a large number of a CSC traits in HNSCC, as it is in lung adenocarcinoma\textsuperscript{57}. One such indication was shown when the downregulation of all known OCT4 isoforms (via RNA interference) in HNSCC cancer stem-like cells of caused an increase in radiosensitivity and a loss of metastatic potential\textsuperscript{70}.
Metastasis is a Significant Step in Cancer Progression

Metastasis marks an especially prominent step in cancer treatment and prognosis. The advent of metastasis may result in a disease that is no longer treatable and causes the majority of cancer-related deaths today. Invasion from the primary tumor into local and distal locations is usually trigged by mutations that modify the expression of just a handful of important genes. In fact, investigators have suggested that the assessment of five chromosomal locations can accurately distinguish a metastatic cell from its primary tumor counterpart.

Epithelial and Neuronal Cadherins

Cadherins (named after “calcium-dependent adhesins”) refer to a class of transmembrane proteins responsible for maintaining cell-to-cell adhesion. These proteins depend on the association of calcium ions to their extracellular Ca2+-binding domains to keep cells anchored against one another. Their functions span beyond that of a “molecular glue,” however, often providing the much needed trigger signal to initiate morphogenesis as one cell binds to another. Cells can influence each other by binding to specific cadherins, which then trigger downstream cascades of signaling molecules and the subsequent transcription factors.

Cells expressing one type of cadherin are thought to cluster amongst themselves to the exclusion of cells that do not express the same type of cadherin. Cells like spinal neurons display high affinity for one another through the interaction of their neuronal cadherin (N-cadherin) while showing decreased attachment to other types of cells such as epithelial cells that are thought to express epithelial cadherin (E-cadherin).
Epithelial-Mesenchymal Transition (EMT)

EMT refers to the theoretical conversion of epithelial cells into mesenchymal cells during embryogenesis. The process is said to be marked by an increase in cell mobility as well as the repression of epithelial cell-to-cell adhesion factors including E-cadherin. The mesenchymal phenotype allows the stem cells near the epithelia to migrate into destinations in the interior of the embryo to achieve proper body formation and development. Notably, this invasive trait of embryonic cells is often highly associated to the induction of oncogenic factors such as Src, Ras, and the Wnt/B-Catenin pathway and may therefore be closely related to cancer progression. In fact, evidence suggests that cancer cells readily express several types of cadherins, the regulation of which can affect cancer cell behavior.

Metastasis as an Imitation of EMT

Similarly to the stem cell theory of cancer, there exists an “EMT theory of metastasis.” Metastasis is often seen as a mutated parallel of EMT. It was shown that cancer cell lines derived from metastatic cancer sometimes show mesenchymal, fibroblastic morphologies regardless of the actual sites of origin or predecessor cell types. Metastatic carcinoma also exhibit a near universal loss of E-cadherin expression, suggesting a loss of epithelial characteristics (typical of many carcinomas) and a transition to a pseudo-mesenchymal state. In addition, there is indication that the transition is also correlated to an increase in N-cadherin expression, which is thought to be associated with gastrulation-related migration. However, the exact role of the N-cadherin in cancer cell adhesion and/or invasive mobility is unclear.
The notion of cancer stem cell EMT is based largely on the histological analyses of cancer cells of epithelial origin that have acquired fibroblastic morphologies. Though there is little dispute about the negative correlation between E-cadherin expression and cancer metastasis in a wide variety of cancers including HNSCC, N-cadherin upregulation was shown to decrease migration in certain cell types. There is also a conflicting report that cancer dissemination may be hindered by N-cadherin expression. In fact, N-cadherin may play a suppressive role in the metastasis of osteosarcoma, with N-cadherin overexpressing cell lines and in vivo biopsies showing distinctively low migration rates. N-cadherin expression was also correlated with low incidence of migration in cervical and liver carcinomas, with investigators consistently discovering low expression of N-cadherin in assays of metastatic tissue. As a cell-to-cell adhesion molecule that binds similar cells together, it is possible that N-cadherin is promoting tumor integrity similarly to the way it encourages attachment during embryogenesis. During neural crest formation, the migrating epithelial cells need to downregulate N-cadherin to reduce binding and to initiate proper neurulation.

Regardless of the uncertainties, the EMT theory of metastasis is a logical extension of the stem cell theory of cancer. If cancer cells are the malignant imitations of stem cells, they may very well migrate in a similar fashion that stem cells do.
METHODS AND RESULTS

Cell Culturing and Maintenance

The cancer cells used in this investigation were grown in a 50:50 mixture of DMEM/F12 media (Mediatech – Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. The normal human oral keratinocytes and OKF6/Tert1 immortalized keratinocytes were grown in EpiLife Media (Life Technologies – Grand Island, NY) supplemented with the EDGS epidermal growth factors and penicillin-streptomycin. All cells were grown in sterile, humidified incubators at 37 degree Celsius and 5% CO₂ concentration.

Detecting OCT4 Isoform Expression

To ascertain the biological significance of OCT4 in head and neck cancer, our group measured the expressions of OCT4A and OCT4B in the normal and cancerous cells of the head and neck as well as the cultures of human embryonic stem cells (hESCs). The detection of the OCT4 isoforms was carried out by protein detection through immunofluorescence (IF), then verified by quantitative reverse-transcriptase PCR (qPCR) of the mRNA transcripts. To conduct IF, the cells were first fixed using pure methanol, then blotted using approximately 1ug of each primary antibody. For qPCR, mRNA was extracted by using the RNeasy kit (Qiagen – Valencia, CA) then treated with RQ1 RNase free DNase (Promega – Madison, WI) to eliminate any DNA contamination. The mRNA was then converted to complementary cDNA using the iScript Reverse Transcriptase (Bio Rad – Hercules, CA).
The lack of a commercially available OCT4B antibody led to improvisations in the detection method. Two antibodies were used: one mouse antibody (Millipore – Billerica, MA) recognizes an epitope in the N-terminus region specific to OCT4A, while the other rabbit antibody (Abcam – Cambridge, MA) recognizes the universal C-terminus epitope. After associating the OCT4A specific antibody to rhodamine and the universal antibody to fluorescein, we determined that regions of tissue or cell culture that shows an equal overlap of red and green fluorescence (and therefore showing yellow coloration in the compounded image) must be expressing the OCT4A isoform. On the other hand, regions that are predominantly green must be expressing solely the OCT4B isoform. We did not discover any regions that were predominantly red, which helped confirm the specificity of the two antibodies.

qPCR was carried out using the SYBR Green I Taq polymerase and the Lightcycler 480 temperature cycler (Roche – Basel, Switzerland). A pair of primers specific to the unique exon 1 region Oct4A mRNA was used to detect its expression. A second pair of primers specific to the Oct4B mRNA exon 1 was used to detect Oct4B expression. The expression of Beta-2 microglobulin (B2m) was measured to serve as the positive control.

**OCT4A is Prevalent only in Embryonic Stem Cells**

IF results show that the H1 embryonic stem cell line shows a high level of OCT4A expression at day 2. The OCT4A concentration decreases until day 10 once the cells have committed into specific lineages and formed into embryoid bodies (Figure 3). At this stage, the cells are thought to suppress OCT4 to allow differentiation and the formation of embryonic germ layers. Regardless of stage, hESCs do not show regions that were predominantly green, indicating a lack of OCT4B expression.
In normal human oral keratinocytes (NHOKs), OCT4A fluorescence was minimal, suggesting no protein expression. OKF6/Tert1 immortalized keratinocyte cell cultures show similarly low OCT4A expression (Figure 4).

In head and neck cancer cell lines, OCT4A is largely absent (Figure 5). Four lines (OSCC1, OSCC4, UMSCC5, and UMSCC17B) show low expression of OCT4A in IF, though the OCT4A fluorescence is overshadowed by the OCT4B fluorescence in the compounded image. The qPCR results also suggest that OCT4A is not normally expressed, as only UMSCC1 cells show a significant pattern of Oct4A transcription (Figure 6).

**OCT4B is Readily Expressed in Neck Cancer**

Unlike OCT4A, OCT4B is highly expressed in HNSCC regardless of the cell line. This is validated by the high levels of the Oct4B transcript as shown through qPCR (Figure 6). Overall, Oct4B transcription was found to be least 2-fold greater in head and neck cancer cells than in normal cells. Though the Oct4A transcript was detected in the UMSCC1 cell line, it did not seem to result in actual protein expression, as determined by IF (Figure 6). This may be due to post-transcriptional modifications that downregulates the translation of the Oct4A mRNA.

The two normal keratinocyte lines did not express OCT4B (Figure 4). Interestingly, the immortalized OKF6/Tert1 keratinocytes expressed small amounts of OCT4B. This may be a result of unregulated cell cycle, as OKF6/Tert1 cell immortality is induced by deregulation of the telomerase reverse transcriptase (Tert) gene in an otherwise normal oral keratinocyte.⁹⁹
In vivo Significance of OCT4 Isoform Expression

The in vivo significance of our findings in cell lines was confirmed through IF of paraffin-preserved patient biopsies. Similar IF techniques were carried out after dissolving the paraffin blocks in xylene, then briefly washing with increasingly lower concentrations of ethanol from 100% to 50%. To encourage antibody penetration, the biopsies were finally washed with water for 3 minutes then treated in a citric acid buffer at 85 degrees Celsius for 30 minutes.

In normal tissue, neither OCT4A nor OCT4B expression was detected (Figure 7). However, OCT4B was found to be the predominant isoform in HNSCC as OCT4A was not expressed (Figure 8). The results in patient samples generally correlated with the findings from cell lines.

The malignancy of the OCT4B expressing cells were verified by confirming their morphology through Hematoxylin-Eosin (HE) staining (Figure 9). The biopsies used for IF were washed briefly with double distilled water then stained with hematoxylin. The tissues were counterstained with eosin Y, then washed with increasingly lower concentrations of ethanol from 100% to 50% to remove residue.

Overexpressing OCT4A in HNSCC

To gauge the function of OCT4A in HNSCC, our group increased OCT4A expression in the UMSCC2 cell line through plasmid transfection. The OCT4A isoform was chosen because of its likely roles in stem cells, and the likelihood that stem cell pathways lead to a more malignant phenotype. In addition, the OCT4B isoform is already highly expressed in cancer cells, making its overexpression assay unnecessary. The UMSCC2 cell line was chosen because of two

---

a Courtesy of Dr. Reuben Kim, UCLA School of Dentistry.
reasons. First, it was derived from a non-metastatic tumor and therefore should not already express the metastasis-related traits like invasion. Second, it was found to express a significant amount of OCT4B but not OCT4A, which would allow for a proper assessment of the effects of OCT4A through its overexpression.

Overexpression plasmids (Figure 2) containing the Oct4A coding region as well as the G418 resistance gene were obtained (Origene – Rockville, MD), then transfected into 70–80% confluent UMSCC2 cells cultures using the LT1 lipid complex (Mirius Bio – Madison, WI). After growing the cultures for 3 hours in an antibiotic-free media, a 1 to 3 ratio mixture of plasmid and LT1 (respectively) were introduced into each plate. The cells were then selected with 400ng/ml G418 media over a course of two weeks to establish stably expressing clones. A plasmid containing the GFP gene instead of the Oct4A coding region was used as the control vector. The GFP-transfected cells were grown in similar conditions and their expression was confirmed using a fluorescent microscope.

The expression of the Oct4A gene was verified by rtPCR (Figure 10). The expression of Oct4A was found to be higher in 3 of the selected UM2-OCT4A clones (named UM2-OCT4A1, UM2-OCT4A2, and UM2-OCT4A5) but similarly absent in both the parental and the two control-vector clones (UM2-GFP1, UM2-GFP3).

**OCT4A Encourages Proliferation**

The three OCT4A overexpressing clones were grown in a 12-well plate. Each row of four wells was seeded with 10,000 cells of one of the clones, and allowed to grow over a course of 4 days. At each subsequent day, one well of each clone was collected then counted through trypan blue dye exclusion assay using a cell viability analyzer (Beckman Coulter – Brea, CA). The two
GFP control clones and the parental cells were seeded similarly on a second 12-well plate then counted in conjunction with the OCT4A overexpressing clones. The growth rates of all 6 cell lines were plotted and their values were matched to an exponential equation using Microsoft Excel.

Overall, the OCT4A clones were found to grow significantly faster than the GFP-control clones or the parental cell line (Table 1). On the fourth day, a well containing the UM2-OCT4A5 clone was found to have almost 5-fold more cells compared to the wells containing cells that were not expressing OCT4A. The slower growing UM2-OCT4A1 and UM2-OCT4A2 clones grew approximately 2-fold faster than the control counterparts (Figure 11). As expected, the coefficient of determination ($R^2$ value) of the growth rates of each cell line was confirmed to be significant at >0.85.

**Detection of E- and N- Cadherin Expressions**

EMT is one of the hallmarks of stem cell development and embryogenesis as well as cancer metastasis. Therefore, our group searched for indications that the expression of OCT4A, a stem cell factor, may induce EMT-related processes when expressed in head and neck cancer. Since Islam et. al and Schipper et al have found correlations between the expression of the different cadherins and head and neck cancer metastasis, we measured the changes in expression levels of the E- and N-cadherin transcripts in both parental and OCT4A overexpressing OSCC cell cultures.

Pellets were collected from cultures of UMSCC2 parental, GFP control, and OCT4A cell lines. After briefly washing the cells in DPBS (Mediatech – Manassas, VA) to completely remove media, the pellets were treated in the same manner with the RNeasy kit, the RQ1 DNase,
and the iScript Reverse Transcriptase to create cDNA from their mRNA transcripts. Two pairs of primers, one specific for the E-cadherin transcript and another specific for the N-cadherin transcript, were used to detect cadherin expression through reverse-transcriptase PCR (rtPCR).

**E- and N- cadherin transcripts are Downregulated in HNSCC that overexpress OCT4A**

The rtPCR products were assayed using electrophoresis in a 2% agarose gel containing ethidium bromide. Upon exposure to ultraviolet light, gel results showed that the UM2 parental and GFP control cells expressed E- and N-cadherins natively (Figure 12). However, the expression of both of the cadherins were absent in OCT4A overexpressing cell lines.

**Treatment of HNSCC Cells with Cisplatin**

Another characteristic commonly attributed to cancer stem cells is the resistance to chemotherapeutic treatments. To gauge the viability of OCT4A expressing cells in chemotherapeutic solutions compared to those that do not, we treated the cell lines with 13 uM of cisplatin over the course of 48 hours then collected them for cell counting. The 13 uM concentration was determined by treating UM2 parental cell lines with 5, 10, 15, and 20 uM concentrations of cisplatin. It was found that concentrations below 10 uM did not cause significant amounts of apoptosis, while anything above a 15 uM concentration seemed to cause too much.

Cells from UM2-OCT4A1, UM2-GFP3, and UMSCC2 Parental lines were seeded. Each cell line was seeded across three wells of a 12 well plate, with each well containing 100,000 cells. The cells were then treated with 13uM of cisplatin each. Each plate was collected at 24 hour intervals to determine the number of living cells.
OCT4A Overexpression Increases Viability of HNSCC in Cisplatin

Cisplatin treatment reduced cell numbers across cell lines (Figure 13). However, the viability of cells overexpressing OCT4A was found to be significantly greater than those that did not. After 1 day, the UM2-OCT4A1 line had over 25% higher viability compared to the viabilities of the other two cell lines. At 2 days, the OCT4A overexpressing line had almost 50,000 viable cells, compared to less than 20,000 for the other two cultures. The numbers of living cells in the OCT4A line was relatively constant over time, with smaller decreases at each 24 hour interval.

Migration Potentials of HNSCC

Though the downregulation of E-cadherin indicates a more invasive phenotype, we conducted a scar-healing assay to gauge the effect of OCT4A overexpression on migratory potential. To produce a comparison between OCT4A expression and absence, we grew three cell lines (UM2-OCT4A1, UM2-GFP1, and UM2 parental), each in its own 60mm plate. The cells were first grown to over 90% confluency in a typical 10% FBS medium, then placed in a 1% FBS medium to prevent further division. Artificial “scars” were produced using the tip of a 200ul pipet. The cells’ migration into the previously scarred areas was monitored over the course of two days.

OCT4A May Not Affect Migration

Surprisingly, the overexpression of OCT4A did not seem to accelerate migration into the scar area of the 60mm plates. All three cultures did not show notable migration, even after 36 hours (data not shown). Many of the cells began dying after 48 hours, perhaps as a result of
serum deprivation. However, this may be consistent with the observation that OCT4 levels in stem cells decrease in response to lower concentration of serum\textsuperscript{100}. In embryonic stem cells, it was found that a decrease in serum levels led to a decrease in OCT4 expression, which in turn allowed the stem cells to differentiate\textsuperscript{100}. Though this requires further investigation to ascertain, it is possible that the decrease in serum levels led to downregulation of the transfected Oct4A gene through epigenetic or post-transcriptional mechanisms. The downregulation of OCT4 in the UM2-OCT4A1 line may reduce its OCT4A expression to a level similar to those found in the UM2-GFP1 and the UM2 parental lines, thereby nullifying many of the downstream effects of OCT4A.
CONCLUSIONS

Though OCT4A was found to be rarely expressed in cancer cell lines as well as in patient cancer tissue, our data indicate that OCT4A could have roles in cancer. Its expression is linked to characteristics such as proliferation, viability in the presence of cisplatin, and decreased cadherin expression. On the other hand, the expression of OCT4B was found to be relatively constant.

OCT4A as a Possible Factor behind Aggressiveness

The traits positively modulated by OCT4A suggest that OCT4A expression may make an existing cancer more aggressive. First and foremost, increased proliferation is an obvious contributor to larger tumor size. Tumors are increasingly damaging as they increase in volume, and larger tumors are more likely to metastasize as they break through the surrounding layers of neighboring tissue. Once the cells do metastasize, they will also be able to grow into visible tumors in a shorter period of time.

In addition, decreased expression of E-cadherin is a well-established marker of cancer cell mobility. E-cadherin loss can significantly dislodge epithelial cancer cells like head and neck cancer cells from one another and encourage spread. In addition, the loss of E-cadherin has also been associated with a more mesenchymal phenotype, which allows for a more streamlined intrusion into the circulatory and/or lymphatic system.

Furthermore, increased viability despite cisplatin treatment is a significant indicator of poorer prognosis. As cisplatin (and possibly other types of chemotherapeutic agents) become less and less effective at completely eliminating the cells of a tumor, the likelihood that few or more
cells will remain alive even after a full-course chemotherapy regimen is concluded. This in turn increases the likelihood of recurrence.

**OCT4B as a Putative Downstream Target of hTERT**

We found that NHOK do not express OCT4B while their slightly mutant counterparts, the OKF6/Tert1 cells, express noticeable amounts of the isoform. Theoretically, the only difference between the two lines is that only the OKF6 line exhibits heterozygous overexpression of hTert. This may be an indication that OCT4B is one of the downstream targets of hTert. The overexpression of hTERT in keratinocytes seems to result in OCT4B expression while unmanipulated cells do not express OCT4B. The presence of OCT4B in HNSCC could be explained by the fact that, in a comprehensive analysis of HNSCC, over 80% of the samples were found to have elevated levels of hTERT activity. If proven true, hTERT may be an interesting inducer to one isoform of OCT4 but unrelated to the other.

**OCT4A May Encourage Tumor Progression through E-cadherin Downregulation**

The inverse correlation between E-cadherin and Oct4A transcription in our UM2-OCT4A cell lines suggests that head and neck cancer may utilize OCT4A expression to promote EMT. One of the hallmarks of embryonic EMT is the downregulation of E-cadherin. In turn, this downregulation allows for the transition of static, epithelial cells into mobile, mesenchymal cells. Similar cases of E-cadherin in increasing cancer cell mobility has been documented in a wide variety of tumors (including those of the head and neck), which has led to the notion of EMT as a mechanism for cancer cell metastasis. In accordance to this model, we find it possible that
OCT4A overexpression may contribute to the EMT process in HNSCC. OCT4A overexpression has been correlated to a decrease in the transcription of the E-cadherin gene, and in turn, we suspect that the UM2-OCT4A cells are likely to express other traits associated with the loss of E-cadherin expression. Though our scar healing assay proved inconclusive, the positive correlation between OCT4A and migration found in other types of carcinomas suggest that there may have been a third factor (namely serum deprivation) that interfered with OCT4 expression in the UM2-OCT4A cells.

The N-cadherin Uncertainty

Though the upregulation of N-cadherin is sometimes regarded as a marker of metastasis, we found that the at least 1 in 8 (12.5%) of HNSCC cell lines grown natively expressed N-cadherin. And like E-cadherin, N-cadherin was readily downregulated upon introduction of OCT4A. In the case of cells where N-cadherin is already a component of the plasma membrane, these molecules may be functioning more as adhesion molecules than metastatic indicators. Kashima et. al and Asano et al. have already shown that N-cadherin expression leads to decreased cancer cell spread in two different types of cancers (osteosarcoma and astrocytoma, respectively) that originate from N-cadherin rich tissue. The mutual binding activity of healthy, N-cadherin expressing cells to one another is also well known, and N-cadherin on the surfaces of UMSCC2 cells may be another reason behind the round, tightly bound morphology of parental UMSCC2 colonies.
DISCUSSIONS AND IMPLICATIONS

So far, most researchers do not realize the existence of multiple isoforms OCT4. This leads to confusing contradictions as one investigation may conclude that cancer cells do not express OCT4, while another may state that OCT4 is in fact prevalent. There is even less distinction between the OCT4 isoforms in the field of head and neck cancer research; we have found several publications that detail the role of OCT4 in cancer, but only one that successfully distinguishes the existence of the OCT4A and OCT4B proteins. Our research allays that confusion and recognizes that the two isoforms are differentially expressed in HNSCC. We have shown that the expression of OCT4A can mark an important step forward in cancer progression while OCT4B is constitutively active in HNSCC.

The identification of OCT4A but not OCT4B as a putative cancer stem cell can be the next significant factor in isolating cancer stem cells with absolute certainty. The detection and targeting of cancer stem cells is a significant hurdle for therapy because most of the traditional treatment methods are ineffective towards these self-renewing populations. Unfortunately, the therapy-resistant cancer stem cell populations are also more malignant, invasive, and tumorigenic, which may account for the large number of recurrences observed in HNSCC cases worldwide. A more directed approach that acts against the specific mechanisms of the cancer stem cell is necessary.

The next logical pursuit of this study would be the identification of OCT4A targeting molecules that can selectively suppress the actions of OCT4A expressing cancer stem cells. Doing so would require the usage of our OCT4A overexpressing UMSCC2 cell lines, and the search for factors that can downregulate OCT4A in healthy stem cells. One promising candidate
is the tumor suppressor molecule CDK2AP1, which was shown to decrease OCT4 activity in human embryonic stem cells.

It would also be logical to better detail the role of OCT4B in both cancer and non-cancer cells, as OCT4B was detected in immortal cells of both types. Overexpression assays of OCT4B in cells that do not express OCT4B could help determine its exact role in cell cycle regulation. The downregulation of OCT4B in non-stem cancer cells can also shed light on the role of OCT4B in the maintenance of malignant cells. Since OCT4A and OCT4B are alternatively translated products of the same gene, it is possible that cancer cells switch between the expression of the two isoforms to alternate between a metastatic and a static phenotype. In fact, Hu et al. have found that an shRNA induced downregulation of an unspecified OCT4 isoform in MCF-7 breast cancer cells leads to increased EMT initiation, while Wang et al. state that MCF-7 cells do not express OCT4A.

Furthermore, it may be beneficial to further verify the epigenetic regulation of OCT4A and OCT4B. Our preliminary analysis of the OCT4 gene and the upstream regulatory regions shows that it contains a single CpG island between Exon1 of Oct4A and Exon1a of Oct4B. Wang et al. have also shown that the Oct4 promoter and regulatory regions are normally highly methylated in breast cancer cells, which suggests that a non-epigenetic mechanism may play a role in OCT4 expression as well. In this view, the implication between hTERT and OCT4B may also be important. hTERT levels are only correlated with OCT4B levels. In addition, hTERT expression may provide a mechanism through which cells upregulate OCT4B without affecting OCT4A expression.

Lastly, we would like to see a more comprehensive effect of OCT4A overexpression on cancer cell migration. Instead of using low-serum media which may have unintentionally
downregulated OCT4A expression, we may find an alternative in specially formulated serum-free media. We may also see interesting results in direct invasion assays such as the transwell assay or matrigel invasion assay, which can provide clues as to how well the OCT4A overexpressing cells can penetrate through solid tissue-like substances.
**Figure 1:** The Human OCT4 gene is alternatively spliced to form two highly similar but differentially sized isoforms. The two isoforms share four exons.
Figure 2: The Oct4A Coding Plasmid includes the Oct4A coding/exon regions paired to a CMV promoter. The Oct4A gene is tailed by a Myc tag and a PolyA signal. The plasmid also includes a Neomycin/G418 resistance gene for use in selection.
Figure 3: OCT4 Isoforms in hESC show that OCT4A is the predominant isoform in embryonic stem cells until the cells have differentiated at 10 days.
Figure 4: OCT4 Isoforms in Non-cancer Oral Keratinocytes IF of the non-cancer oral keratinocyte cell lines show that neither isoform is found in the normal keratinocytes. However, OKF6/Tert1 cell lines show significant OCT4B expression.
Figure 5: OCT4 Isoforms in HNSCC unlike the case in non-cancer cells, OCT4B expression predominates the IF assays of head and neck cancer cell lines.
Figure 6: The Quantitative PCR of Oct4 Isoform Expression correlate well with the IF, though small discrepancies leave the door open for the possibilities of post-transcriptional regulatory mechanisms that selectively modulate each isoform’s expression.
Figure 7: OCT4 Isoforms are missing in Normal Tissue as it is in normal cell lines. Despite the high number of cells (as evident in the DAPI images), both the red and the green fluorescence are remarkably dark.
Figure 8: OCT4 Isoform Expression in Cancer Tissue is significant as in vivo tissue show only OCT4B expression as well.
Figure 9: H&E Stain Images of Cancer Tissue show that the tissues assayed in Figure 8 are indeed cancerous, with uncertain morphologies and poorly defined cell borders.
Figure 10: Verification of Oct4A Expression was carried out by rtPCR with Beta-2-microglobulin as the loading control. NHOK served as the negative control hESC as the positive control.
Table 1: Numbers of Transfected UMSCC2 Cells suggest that OCT4A overexpression allows cells to grow at a much more rapid rate. Cells were counted using a viability analyzer that utilizes the trypan blue exclusion method.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Days Since Seeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>OCT4A1</td>
<td>10000</td>
</tr>
<tr>
<td>OCT4A2</td>
<td>10000</td>
</tr>
<tr>
<td>OCT4A5</td>
<td>10000</td>
</tr>
<tr>
<td>GFP CTRL1</td>
<td>10000</td>
</tr>
<tr>
<td>GFP CTRL2</td>
<td>10000</td>
</tr>
<tr>
<td>Parental</td>
<td>10000</td>
</tr>
</tbody>
</table>
Figure 11: UMSCC2 Cell Growth over 4 Days graph shows that the growth is indeed higher, but that it still fits an exponential growth pattern. The growth curve of the control and parental cell lines show largely similar courses, while the curves of the OCT4A expressing lines climb much higher.
Figure 12: Cadherin Expressions in HNSCC indicate that OCT4A expression downregulates the transcription of the E- and N- cadherin genes.
Figure 13: UMSCC2 Cell Viability after 13μM Cisplatin Treatment is notably increased in the OCT4A expressing line than in the control and parental lines.
References


