Devil Facial Tumor Disease, A Potential Model of the Cancer Stem-Cell Process?

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***Abstract*—Tasmanian devil facial tumor disease (DFTD) is a naturally occurring contagious cancer which is transmitted as a clonal cell line between devils. The malignant cell line evolved from a Schwann cell or precursor prior to 1996 and since then has undergone continuous division without exhausting its replicative potential, suggesting a profound capacity for self renewal. It is therefore important to elucidate whether DFTD may have a stem cell origin. Deciphering the pathways regulating DFT cell proliferation and survival could lead to increased understanding of this transimissible cancer and to the development of successful therapies to halt the disease. We investigated whether DFT cells have originated from transformed stem cells by measuring the expression levels of thirteen genes characteristic to embryonic stem and/or pluripotent germ cells. No differences in gene expression were observed between DFT cells and peripheral nerve controls, and therefore our results provide additional support for Schwann cell or peripheral nerve origin of DFTD. Although our dataset is preliminary, it does not suggest that DFTs have cancer stem cells (CSCs) origin. We provide details of further experiments needed to ultimately confirm the role of cancer stem cells in DFTD progression.**

***Index Terms*— cancer, marsupial, Stem cell, Tasmanian devil**

I. Introduction

Malignant tumors have been described as heterogeneous populations of cells, where cells with different biological functions compete for resources within the microenvironment of the neoplasm [1-3]. Cells with the highest proliferative potential, immune evasion capacity and adaptability have the highest chance of survival and hence confer significant evolutionary benefits [1]. The mechanism underlying cancer evolution has long been of interest, and two mutually non-exclusive, but concurrent theories have been developed to describe neoplasm progression:

(i) The stochastic or clonal evolutionary theory posits that during tumor development a transformed cell or cells gain unlimited proliferative capacity, and hence produce uncontrolled cell growth. The subsequent accumulation of random mutations result in a heterogeneous cell subpopulations within the tumor, and the concomitant selection of sub clones drives tumor evolution [4]. Importantly, according to the stochastic or clonal evolutionary theory any of the cancer cells can participate in tumor growth, development and recurrence [5].

(ii) A recently resurrected theory, the hierarchical model or cancer stem cell (CSC) hypothesis [6, 7] also traces tumor origins to single mutated cells with unlimited proliferative potential, but in contrast to the clonal model, the cells possess stem cell qualities [8]. The concept of CSCs

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assumes that they arise from transformed stem cells (either tissue stem cells or their immediate progeny) via accumulation of genetic modifications (mutations and epigenetic alterations) [9], or from de-differentiation of somatic cells via acquiring stem cell characteristics [10], and the development of the tumor results from the clonal evolution of the CSC population [11]. The multipotent nature of these cells results in cellular heterogeneity within a tumor. As a result of hierarchical differentiation, the tumors contain cellular subcomponents that retain key stem cell properties, but the majority of the progeny cells do not possess self-renewal potentials and hence do not contribute to tumor progression [12]. In contrast to the clonal evolution theory, the cancer stem cell hypothesis postulates that only the CSCs are responsible for tumor growth, participate in tumor progression, drive metastasis and tumor reoccurrence [13]. Recently several human and animal studies have reported evidence that cancer stem cells are responsibly for the growth of certain brain, skin, intestinal and bone tumors [14-18]. Although both evolutionary theories have attracted ample advocates and critics [6, 13, 19, 20], the validity of either still remains to be resolved.

A recent paper by O'Neill [21] suggested that transmissible animal tumors might have originated from stem cells and hence provide unique models to study cancer stem-cell processes. Canine transmissible venereal tumor (CTVT) and Tasmanian devil facial tumor (DFT) are two, naturally occurring clonally transmissible cancers [22]. The two diseases produced by these transmissible cancers share similar etiology; both of them originated from a rogue cell-line, and can be transplanted as allografts between unrelated hosts by physical transfer. CTVT is a globally distributed sexually transmitted tumor of dogs. The disease possibly arose thousands of years ago from a single wolf, and therefore CTVT is considered to be the oldest known somatic cell line [23, 24]. Devil Facial Tumor Disease (DFTD) is a more recently emerged infectious disease of Tasmania devils, the world largest remaining carnivorous marsupial [22, 25, 26]. The first case of DFTD was observed in 1996 in the north-eastern Tasmania [25] where animals were sighted showing some of the hallmark gross pathologies associated with what was later defined as DFTD. The disease is characterized by large ulcerating lesions around the mouth and the face of the affected animals [27]. This aggressive cancer is transmitted by biting between the devils during sexual and feeding interactions [28]. DFTD indiscriminately affects both female and male devils and generally causes death within six month of the appearance of initial lesions. Due to the rapid progression and transmission of the disease, the Tasmanian devil population has declined by 80% over the past 15 years and may face extinction in the wild within 25-35 years [25, 29]. Cytogenetic analyses have revealed that DFTD is caused by a rogue cell line [26] most likely originated from Schwann cells of the peripheral nerve sheath [27, 30]. Devil Facial Tumor (DFT) cells possess a highly rearranged genome, characterized by tumor-specific complex translocations and chromosomal rearrangements [26, 31]. The clonal nature of DFTs have been supported by both large-scale genetic [30, 32] and immunohistological [27] analyses. Furthermore, the analyses of microsatellite markers and functionally important genes, such as the Major Histocompatibility Complex (MHC) revealed that DFT cells in different individuals are genetically identical, demonstrating the stable nature of the tumor [22, 30, 32-34]. However, three recent studies have described the existence of four, closely related but karyotypically distinct DFT strains, suggesting that the tumor is clonally evolving via stepwise mutational changes [32, 35, 36]. Since their emergence in 1996 [26] DFT cells have undergone continuous division and propagation in thousand's of devils without exhausting their replicative potential, suggesting an apparent capacity of self renewal. Here we provide preliminary data on the possible existence of CSCs in devil facial tumors and discuss the necessary experiments to test whether a subpopulation of self-renewing CSCs exists in DFTD.

II. Materials and Methods

A. RNA extraction and quantifying gene expression by quantitative RT-PCR

RNA was extracted from four spleen, seven peripheral nerve and nine primary tumor samples using a combination of Trizol (Sigma, St. Louis, MO) and Qiagen RNeasy mini kit (Qiagen, Germantown, MD). RNA quality and quantity were measured on an Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA). Genomic DNA was removed from the RNA samples by the DNAse I AMPD1 kit (Sigma, St. Louis, MO) and cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD). Thirteen genes (*AK3*, *BMI1*, *DPPA4*, *FGF14*, *FZD7*, *GABRB3*, *GGTLA5*, *GPC4*, *NANOG*, *Oct-4*, *RB1*, *SCNN1A*, *SOX2*) previously demonstrated to be significantly expressed in embryonic stem cells and human pluripotent germ cell tumors [34-36] were selected for quantitative real-time PCR analyses (Table 1). Gene-specific primers spanning across exon boundaries were designed based on the Tasmanian Devil genome annotation (Ensembl [29]), using the Primer3Plus website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 1). Two genes, *GAPDH* and *GUSB* were used as normaliser genes following the description of Murchison et al. [19, 27] (Table 1). The Q-PCR reactions were performed on the RotorGene6000 (Qiagen, Germantown, MD) in 15 µl total volume, containing 7.5 µl of Qiagen 2xQuantifast Sybr Green PCR master mix (Qiagen, Germantown, MD), 0.25-0.5 µM forward and reverse primers (optimal primer concentrations were established for each primer

combinations) and 1 µl of cDNA (5ng/µl concentration). Reverse transcriptase negative and cDNA negative samples were run alongside the cDNA samples as controls to detect genomic DNA contamination and primer-dimer formations. Q-PCR conditions were established according to the manufacturer protocol: 95º C for 5 min denaturation followed by 40 cycles of 95º C for 15 s and 60º C for 30 s (annealing temperature, AT). Fluorescence signal was acquired at the AT. To evaluate the specific amplification a final melting curve analysis (from AT up to 99° C) was added under continuous fluorescence measurements.

B. Statistical analyses

Relative quantifications of gene expressions were performed using sample-crossing points, and data was analysed with the Rotor Gene 6000 software 1.7. (Qiagen, Germantown, MD), applying the "second derivative maximum" method [38]. The Excel application Best-Keeper [39] was used to check the data for statistical significance, normality and reliability, and the normaliser gene GUSB was chosen as reference based on BestKeeper calculations [39]. The program Rest [40] was used to calculate the normalised fold change of the target gene compared to the reference gene. Statistical significance ($P < 0.05$) was determined by a Pair-Wise Fixed Reallocation Randomisation Test© as described by Pfaffl et al. [40].

III. Results

AK3, FGF14, GGT5, GPC4, RB1 and *SOX2* genes were not differentially expressed between DFT and peripheral nerve samples (Table 2.). Due to low expression of the target transcripts, the expression levels of *BMI1, DPPA4, FZD7, GABRB3, NANOG, Oct-4, SCNN1A* were below the linear range of detection, showing that these genes are not expressed in DFT, peripheral nerve and spleen samples. Five genes, *SOX2, FGF14, GPC4, AK3 and GGT5* had significantly higher expression in nerves compared to spleens (Table 2). Compared to spleen, DFT samples showed up-regulation of *SOX2, FGF14, GPC4* genes (Table 2), but *AK3* and *GGT5* genes were not differentially expressed.

IV. Discussion

Up-regulation and expression of four genes (*FGF14, SOX2, GPC4* and *GGT5*, which have previously been shown to be expressed in Schwann cells [37-40]) in both nerve and DFT samples compared to spleen, adds further support for the Schwann cell lineage origin of DFT cells [30]. The expression of *FGF14* in DFT and peripheral nerve cells is not surprising since the protein encoded by this gene is involved in a variety of biological processes (not only restricted to stem cells) such as tissue repair in response to injury, and, hence act as homeostatic factors [41]. Moreover, *FGF14* also constitutes one of the four genes of intracellular fibroblast growth factors (i*FGF*s), which are important for neuronal signal transduction and regulation in the central and peripheral nervous systems [37].

The high expression of *SOX2* in both peripheral nerve and DFT samples is more intriguing. Apart from being a transcriptional factor with an essential role in maintaining self-renewal of undifferentiated embryonic stem cells, *SOX2* has also been found to be a marker of immature and dedifferentiated Schwann cells [38]. This protein is not typically expressed in neural crest stem cells, but is specifically turned on in Schwann cells where it plays a crucial role in maintaining these cells in an immature state [42]. The observed high level of expression of *SOX2* in DFT cells most likely has an important role in maintaining the tumor cells in an undifferentiated state, without undergoing neuronal commitment which would lead to the loss of proliferative capacity [38, 43]. Schwann cell development occurs through a series of transitional embryonic and postnatal phases, regulated by signaling pathways with characteristic gene expression patterns [42]. A study by Murchison et al. [30] showed high expression of genes characteristics of immature fetal (*SOX2*, *c-Jun*), postnatal pro-myelinating (*POU3F, MPZ*) and myelinating Schwann cells (myelin basic protein (*MBP*), transcription factor *SOX10*, structural myelin genes *MPZ*, *PRX*, *PMP22*), but not genes specific to stem cells. The observed gene expression patterns might indicate the presence of heterogeneous cell populations within DFTs – that is Schwann cells at different developmental stages. Another, more likely, explanation is that DFT arose due to disruptions to developmental pathways in Schwann cells, resulting in DFT cells synchronously expressing genes specific to different Schwann-cell stages.

In conclusion, although our dataset is preliminary, the general lack of up-regulation of stem cell–specific genes suggests that DFTD is unlikely to be of stem cell origin. Moreover cytogenetic [26, 35, 36] and genetic evidence [30, 32, 36, 44] also suggest that the progression of DFTD does not support the cancer stem-cell theory but follows a clonal expansion model [31]. DFTD is a stable, clonal, asexually reproducing cell line which undergoes Darwinian evolution [4, 32, 45, 46]. Stepwise, somatic-cell mutations and sequential selection have most likely resulted in adaptation of this somatic pathogen to the tissue microenvironment of Tasmanian devils [46].

However, to univocally confirm or exclude the role of cancer stem cells in DFT progression additional experiments have to be conducted. For example, ectopic expression of Oct4 in tumor cells have been shown to results in dedifferentiation and enhanced CSC-like properties [47, 48].

Elevated expressions of Oct4 and Nanog have been reported in cancer cell lines and/or primary cancers from various human cancers, e.g. adenocarcinoma [49, 50], breast cancer [50] and melanoma [48], and the study of Schreiber et al. [51] suggested that the expression of these genes actually reflected pseudogene activity. Since Nanog and Oct4 work together with other key pluripotent factors (e.g. STAT3, LIF, FOXD3) a more complex gene expression signature analyses (involving several members of the regulatory network) could confirm the origins of observed gene activity (pseudo- vs functioning protein coding genes).

Furthermore, experimental transfer of DFTD cells between devils [52], and xenograft studies in NOD/SCID mice [53] have shown that cells from primary tumor culture or culture of a previously established xenograft can participate in tumor growth and development. In the latter study the authors achieved successful tumor growth by injecting immunocompromised mice with $1x10^5$-$1x10^7$ DFT cells. However, none of these studies attempted to select for CSCs. Therefore, in order to confirm whether DFT have a cancer stem cell origin, an attempt must be made to identify a population of potential cancer stem cells from fresh tumor samples and/or primary cell cultures. Sarcosphere formation and self-renewal assays [18, 54] should be performed followed by gene signature analyses of stem cell markers [54, 55]. Once a possible stem cell population has been identified the ultimate test of CSC behavior would be to evaluate the number of cells required from the enriched CSCs and from primary DFTs required to initiate a tumor when xenotransplanted into immuno-compromised mice. If the isolated stem cells and primary tumor cells have the same ability to initiate tumors then DFTs most likely do not have stem cell origins.

Although our preliminary data indicates DFTs did not have increased expression of stem cell markers compared to normal tissues, the role of CSCs in DFTD remains an unsolved mystery. It is well known that many human and animal cancers are resistant to currently available chemo- and radiotherapies due to the presence of proliferatively quiescent CSCs [10, 56]. Unambiguous identification of a possible CSC origin of DFTD is s crucial step to the development of successful therapies i.e. bypassing the resistance of CSCs [56], or by actively targeting the self-renewal controlling pathways for the successful eradication of CSCs [10], in order to counteract the spread of this devastating disease.

V. References

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TABLE 1. GENES IMPLIED IN CSCS AND CORRESPONDING PRIMER SEQUENCES (ALL OLIGONUCLEOTIDES WERE SYNTHESIZED BY SIGMA).

	Gene name	Function:	Primers (5'- 3')
1	AK3 (Adenylate kinase 3)	Phospohotransferase enzyme with role in cellular energy homeostasis.	F: CAGCGTGAAGATGATAAACCAG R: CTTGAGGCCAGATTATGTTGG
2	BMI1 (B lymphoma Mo-MLV insertion region 1 homolog)	Oncogene. Necessary for efficient self-renewing cell divisions of adult hematopoietic stem cells, and nervous system neural stem cells.	F: GCCCAGCAGCAATGACAG R: GAGGTCCATTGGCAATATCC
5	DPPA4 (FLJ10713, hypothetical protein)	May play a role in maintaining cell pluripotency.	F: TTGCCTACCTGCTCTTTTCC R: TCCATATAGAAAGGCACCTCAAG
4	FGF14 (Fibroblast growth factor)	Mitogenic and cell survival activities, involved in embryonic development, tumor growth and invasion.	F: GGAAGGGCAAGTTATGAAGG R: AGCCTTTGGCACTGTTTCTC
6	FZD7 (Frizzled homolog 7 Drosophila)	Transduction and intercellular transmission of polarity information during tissue morphogenesis and/or in differentiated tissues. Involved in tumorigenesis via the canonical Wnt signaling pathway [57].	F: GCTTTACTTCTTCGGCATGG R: AGTACTGCGAATTGGCTTCG
7	GABRB3 (Gamma-aminobutyric acid receptor subunit beta-3)	Member of the ligand-gated ionic channel family. Serves as receptor for gamma-aminobutrytic acid, a major inhibitory transmitter of the nervous system.	F: CTGCAAGGGCAAAGAATGAC R: TTCCTGGAATCTCCAACACC
8	GGT5 (Gamma-glutamyltransferase-like activity 5)	Member of a gene family that encodes gamma-glutamyl transpeptidase enzymes.	F: TTCCTCAACAGCTCTCTTAGCC R: GCTTTTGTCCCCTGAAGAAG
9	GPC4 (Glypican 4)	May play a role in the control of cell division and growth regulation.	F: TGTTCCAACGTCATGAGAGG R: TGACCGACTCGATGTTAAAGG
11	NANOG	Transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells. Key factor in maintaining pluripotency.	F: TCCATGTGGTCCAAGAACAG R: GCTCCACATTGGAAGGTTTC
12	Oct-4 or POU5F1 (Octamer-binding transcription factor 4 or POU domain, class 5, transcription factor 1)	Critically involved in the self-renewal of undifferentiated embryonic stem cells.	F: GCTCCACATTGGAAGGTTTC R: CCAGCTGCCTCAAAATCTTC
13	RB1 (Retinoblastoma 1)	Tumor suppressor, controls the proliferation, differentiation, and survival of cells, with central role in stem and progenitor cell' biology.	F: AAAAGGCTTGGGAGAATTGG R: AAGGTGAACGGCATCTCATC
14	SCNN1A (Sodium channel, nonvoltage-gated 1)	Nonvoltage-gated, amiloride-sensitive, sodium channels control fluid and electrolyte transport across epithelia in many organs.	F: TGGGGACTGTACCAAGAATG R: TATGCACATCCGCACTTCTG
15	SOX2 (SRY (sex determining region Y)-box 2)	Transcription factor, essential to maintain self-renewal of undifferentiated embryonic stem cells.	Primers from [30]: F: CCGAGTCTTAAAGAGGCAGCAAACTACT R: CTCAGGAGTTGTCAAGGCGGAGAA
16	GUSB (Beta-glucuronidase)	Used as normalizer gene	Primers from [30]: F: CTG CTG CCT ATT ATT TCA AGA C

			R: CAA GAT CCA ATT CAG GCT TAG
17	GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	Used as normalizer gene	Primers from [30]: F: GACTCAACCACGTATTCGGCTC R: ATATGATTCCACCCATGGCAAGTTCAA

TABLE 2. RELATIVE EXPRESSION RESULTS FROM REST ANALYSES. ITERATIONS: 5000, SIGNIFICANCE LEVEL: $P < 0.05$. UP = UP-REGULATED, NDE = NOT DIFFERENTIALLY EXPRESSED.

PERIPHERAL NERVE VS DFT SAMPLES						
Gene	Reaction efficiency	Relative expression	Std. Error	95% Confidence Interval	P	Result
AK3	1.0	0.18	0.01 - 1.89	0.00 - 15.38	0.14	NDE
FGF14	1.0	3.80	0.29 - 40.71	0.05 - 126.48	0.16	NDE
GGT5	0.89	0.15	0.01 - 2.58	0.00 - 19.11	0.10	NDE
GPC4	1.0	0.59	0.07 - 5.63	0.01 - 25.02	0.52	NDE
RB1	1	1.65	0.17 - 16.17	0.02 - 182.09	0.607	NDE
SOX2	1.0	2.01	0.33 - 11.79	0.16 - 78.25	0.32	NDE
PERIPHERAL NERVE VS SPLEEN						
AK3	1.0	7.92	2.29 - 34.92	0.27 - 166.24	0.04	UP
FGF14	1.0	23.61	2.88 - 106.99	0.57 - 610.24	0.01	UP
GGT5	0.89	61.86	11.91 - 295.99	2.09 - 522.59	0.01	UP
GPC4	1.0	72.94	13.98 - 358.79	2.32 - 630.77	0.00	UP
RB1	1.0	3.18	0.79 - 17.92	0.16 - 40.19	0.22	NDE
SOX2	1.0	129.69	22.75 - 1,573.49	2.33 - 3,345.03	0.00	UP
TUMOR VS SPLEEN						
AK3	1.0	1.41	0.06 - 14.01	0.01 - 61.06	0.79	NDE
FGF14	1.0	89.76	6.66 - 1,131.77	0.32 - 2,025.09	0.00	UP
GGT5	0.89	9.53	0.41 - 154.51	0.09 - 1,153.54	0.14	NDE
GPC4	1.0	42.85	4.74 - 281.54	0.72 - 1,776.34	0.01	UP
RB1	1.0	5.24	0.60 - 63.68	0.09 - 217.07	0.201	NDE
SOX2	1.0	260.93	27.11 - 1,975.31	12.86 - 8,481.22	0.00	UP