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Initiation of Cancer: The Journey From Mutations in Somatic Cells to Epigenetic Changes in Tissue-resident VSELs

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Abstract

Multiple theories exist to explain cancer initiation, although a consensus on this is crucial for developing effective therapies. 'Somatic mutation theory' suggests that mutations in somatic cells during DNA repair initiates cancer but this concept has several attached paradoxes. Research efforts to identify quiescent cancer stem cells (CSCs) that survive therapy and result in metastasis and recurrence have remained futile. In solid cancers, CSCs are suggested to appear during epithelial-mesenchymal transition by the dedifferentiation and reprogramming of epithelial cells. Pluripotent and quiescent very small embryonic-like stem cells (VSELs) exist in multiple tissues but remain elusive owing to their small size and scarce nature. VSELs are developmentally connected to primordial germ cells, undergo rare, asymmetrical cell divisions and are responsible for the regular turnover of cells to maintain tissue homeostasis throughout life. VSELs are directly vulnerable to extrinsic endocrine insults because they express gonadal and gonadotropin hormone receptors. VSELs undergo epigenetic changes due to endocrine insults and transform into CSCs. CSCs exhibit genomic instability and develop mutations due to errors during DNA replication while undergoing excessive proliferation and clonal expansion to form spheroids. Thus tissue-resident VSELs offer a connection between extrinsic insults and variations in cancer incidence reported in various body tissues. To conclude, cancer is indeed a stem cell disease with mutations occurring as a consequence. In addition to immunotherapy, targeting mutations, and Lgr5 + organoids for developing new therapeutics, targeting CSCs (epigenetically altered VSELs) by improving their niche and epigenetic status could serve as a promising strategy to treat cancer.

Keywords Cancer · Somatic mutation theory · Cancer stem cells · Very small embryonic-like stem cells (VSELs)

Introduction

Almost 85–90% of cancers affecting solid organs like endometrium, ovary, prostate, skin, breast, colon, lung, pancreas, bladder, liver, kidney and cervix have their origins in the epithelial cells. According to the somatic mutation theory (SMT), a sequential accumulation of somatic driver mutations, in the oncogenes (offer proliferation advantage) or tumor suppressor genes (suppress apoptosis) in an epithelial cell initiates cancer. This genetically mutated cell further undergoes uncontrolled expansion and epithelial-mesenchymal transition (EMT). During EMT, epithelial cells dedifferentiate and further reprogram to provide a ready source of cancer stem cells (CSCs) [1–5]. CSCs express pluripotent markers like SOX2, NANOG and OCT-4 and exhibit characteristic property of resistance to chemo- and radiotherapy. EMT also results in the generation of mesenchymal stem cells (MSCs) with properties like motility, invasiveness & resistance to apoptosis. The MSCs further give rise to fibroblasts, endothelial cells, pericytes, adipocytes, and macrophages in the tumoral microenvironment. These cells mobilize to distant sites and undergo mesenchymal-to-epithelial transition (MET) to produce metastasis with an epithelial phenotype [6, 7]. Personalized medicine is being developed to target specific driver mutations to win the war against cancer.

The above-described and widely accepted view on how cancer initiates from epithelial cells is an outcome of research over several decades, generous funding, and more than 5

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million publications. However, despite multiple clinical studies and systematic reviews, this explanation seems highly improbable from a stem cell point of view. There exists a lack of clarity on (i) how mutations due to DNA damage even occur in an epithelial cell which is a differentiated cell with a limited life span, lacks the ability to divide, on the path to senescence and is replaced on regular basis by stem cells (ii) cellular mechanisms that offer immortality to the somatic (epithelial) cell that acquires a mutation in the oncogenes or tumor suppressor genes and continues to survive and accumulate further mutations over years (decades) are not yet understood (iii) dedifferentiation/transdifferentiation and reprogramming of epithelial cells into pluripotent CSCs are still highly theoretical and could potentially represent misconceptions (iv) MSCs are neither stem cells nor pluripotent, thus lacking the ability to differentiate into multiple cell types of different lineages like endothelial and nerve cells in a tumor. They are stromal cells that provide paracrine support, are multipotent progenitors that can differentiate into osteocytes, chondrocytes, and adipocytes and serve as an excellent source of growth factors and cytokines [8].

War on Cancer has not yet been won [9] possibly because the understanding of underlying patho-mechanisms that drive cancer origin and progression are not yet deciphered correctly. Bhartiya's group recently showed that excessive self-renewal and blocked differentiation of pluripotent, tissue-resident very small embryonic-like stem cells (VSELs) initiate cancer in mice models [10] and that epigenetically altered VSELs and CSCs share similar characteristics [11]. The present article discusses how research has progressed from the concept of SMT with the crucial role of driver somatic mutations (due to errors during DNA repair) to a possible role of epigenetic changes in the tissue-resident VSELs in initiating cancer. Adverse extrinsic insults or punishments of bad lifestyle result in the transition of VSELs into CSCs with genomic instability. Errors occur during DNA replication when CSCs undergo excessive proliferation and clonal expansion resulting in 'Bad Luck' mutations. Thus, genetic changes are possibly a consequence rather than being an initiator of cancer. The present article falls in the category of a 'House of Brick' rather than one more 'Mansion of Straw' as discussed earlier [12, 13]. Hopefully, the discussions included in this review will bring about clarity on how cancer initiates and targeting the cancer-initiating stem cells will help to accomplish the objectives of the Cancer Moonshot program [14].

Somatic Mutations Theory

Multiple theories have been put forth to explain the underlying causes of cancer [15]. Widely accepted, century-old, somatic mutations theory (SMT) was put forth in 1914 by Theodor Boveri. SMT suggests that cancer occurs as the result of the gradual accumulation of 3–8 driver gene mutations in a somatic cell that offer immortality and growth advantage resulting in increased cell proliferation. SMT has been challenged in the recent past by several groups who have admitted failure of genetic basis for cancer initiation after 100 years of research [16–19]. Weinberg [20], one of the main proponents of genetic changes driving cancer, in his article in the journal Cell (upon completion of 40 years), accepted that cancer is possibly not a genetic disease. Soto and Sonenshein group [16, 17] have opposed the concept of somatic genomic changes causing cancer. Studies on more than 10,000 solid cancers suggest that there may be a link with genomic changes but how the genomic changes result in tumorigenesis is still not clear. They also discussed and cited several studies suggesting that somatic mutations are also reported in normal tissues and several benign conditions (discussed ahead) and thus are not a specific change that only results in cancer. Conversely, there are several childhood tumors like medulloblastoma, and neuroblastoma which have very few or no cancer-driving genes [21]. Zero mutations is an interesting challenge to SMT and thus, it may be more correct to think that cancers beget mutations rather than mutations beget cancers as suggested earlier [22]. The role of somatic mutations in cancer formation is of a driver or facilitator is of great interest and was discussed [23]. Besides genetic changes, epigenetic changes may also have a role in driving cancer. Baker [18] pointed out that SMT, as the name suggests, should be accepted as a theory and not as a fact because only this mindset will lead to further progress in the field. It is reported that certain tumors have 77 mutations per million base pairs of DNA, some have 30, 66 or some even have 10,000. A large fraction of these mutations are simply passenger mutations of no consequence and only a small fraction are driver mutations but it remains a challenge how do classify the driver mutations. Greenman et al. [24] reported 73 of 210 tumors with zero mutations in the coding exons. Similarly, some mutations have both oncogenic and tumor-suppressive roles depending on the context. NOTCH1 is an oncogene in leukemia but a tumor suppressor in squamous cell carcinoma. MYC is usually an oncogene but also has the characteristics of a tumor suppressor. To explain the heterogeneity that exists in tumors, there are distinct sub-populations that vary as the tumor progresses from one stage to another and also from patient to patient. Brucher and Jamall [19] also discussed that mutations are possibly epiphenomenon-post-carcinogenesis events. Generally, scientific articles describe mutations as associations but are misinterpreted as if they initiate cancer. Importantly, it has not been possible to show appreciable clinical benefit by targeting mutations over the past many decades. The authors [25] have very nicely described the timeline of how the SMT theory has evolved over a century. Various paradoxes surrounding the SMT for being the underlying cause to initiate cancer have

been discussed by several other groups as well [18, 25–27] and are listed below.

- Cancer cells display a wide variety of somatic 'driver' mutations with huge intratumor differences which at times, disappear as cancer progresses. Substantial variation in number and pattern of somatic mutations in individual cancers was reported when they were first reported [25]. Their causative role is not clearly understood and are possibly only associations. The existing paradox between mutations and cancer incidence was discussed by Chanock [27]. A large number of mutations occur in the skin which is directly exposed to strong mutagen (UV light) compared to the esophagus, but surprisingly driver mutations in NOTCH1 and TP53 are more common in the esophageal epithelium. Despite this, the risk of developing esophageal carcinoma is much lower than would be predicted based on mutations in NOTCH1 and TP53 [28]. This suggested that besides somatic mutations, additional factors are required to initiate cancer or prevent it from developing.
- Kato et al. [29] discussed that genomic alterations such as those in BRAF, RAS, EGFR, HER2, FGFR3, PIK3CA, TP53, CDKN2A, and NF1/2, considered hallmark drivers of specific cancers are also present in benign and premalignant conditions, at higher frequencies than in their malignant counterparts. Normal tissues also harbor somatic mutations in the cancer genes and hotspots which makes it difficult to accept SMT to initiate cancer [30–32]. Many cancer-associated mutations are also reported in benign diseases like endometriosis [33]. It is indeed intriguing why no clear pattern of mutations has emerged yet for different types of cancers despite several years of efforts [34]. A huge amount of heterogeneity in genetic changes exists even amongst patients with similar types of cancer. Ceaseless genomic changes occur across time within the same primary and metastatic tumor and have broken the hope of a personalized treatment based only on genomic fingerprint [35]. This was recently highlighted in the TRACERx studies (discussed ahead in details) on lung cancer [36] wherein authors concluded that a universal cure for advanced cancer may not be possible since cancers have infinite ability to evolve. Authors closely studied the genetic evolution of lung cancer over 9–10 years and eventually realized that it is impossible to target mutations to achieve a universal cure for lung cancer.
- According to SMT, carcinogenesis requires the multi-step accumulation of DNA changes. These DNA changes not only include mutations in coding regions of genes, but also mutations in non-protein-coding DNA (which represents over 98% of the DNA), epigenetic changes, and aneuploidy. All these DNA changes have a marked effect on gene expression and are very common in cancer samples. It is unclear, however, what causes these DNA changes, in what type of cell they take place, and if they are sufficient to initiate cancer [37]. Moreover, the somatic cells are continuously replaced by the tissue-resident stem cells, then how do they accumulate mutations over the years/decades?
- The cell in which mutations occur initially - still remains elusive. It is known that initial mutations exist in only a limited percentage of cancer cells, they are lost as the disease progresses and a significant proportion of neoplastic cells do not show any mutated genes. All this suggests that mutations may not be necessary for cancer evolution [38, 39]. 2–8 sequential genetic hits develop over 2–3 decades according to SMT and result in cancer initiation. But this concept is challenged by cancers that occur with zero mutations [18, 21]. Reports exist suggesting neither gene mutations nor epigenetic changes were detected in certain tumors even though diverse technologies were used for the studies. Certain pediatric tumors such as medulloblastomas, neuroblastomas, and rhabdoid tumors exhibit zero to two gene mutations [21]. There are several other studies cited in this editorial that have reported zero mutations in the coding regions of proteins in various cancers including ependymoma brain tumors [21]. Currently, 80% of patients with non-small cell lung cancer do not have an actionable driver mutation. Mechanisms underlying the initiation of cancers with zero mutations need to be investigated. Can epigenetic modifications alone without gene mutations suffice to drive cancer?
- There is no known proven set of mutations that can transform a normal cell to a cancerous state. Artificial ectopic overexpression of a few select genes does not promote the neoplastic transformation of normal cells [40]. Mutated oncogenes or cellular growth regulatory genes fail to immortalize human epithelial cells [41]. Moreover, some mutations have both tumor-suppressive and oncogenic roles e.g. NOTCH1 is an oncogene in leukemia and tumor suppressor in squamous cell cancer [42]. Similarly, Myc known for its active role as an oncogene also acts as a tumor suppressor [43, 44]. Possibly the existing concept that only driver mutations facilitate tumor formation may not be true and these may be mere associations/symptoms/consequences of cancer and have no role in cancer initiation [45, 46].
- Many powerful carcinogens do not have mutagenic activity implying they are not genotoxic like endocrine disrupting chemicals (EDCs) and heavy metals. EDCs are synthetic chemicals used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides. EDCs display estrogenic and androgenic effects, and their exposure has been linked to increased cancer risk including those of the thyroid, breast, and prostate

[47]. Cancer incidence is also known to increase upon exposure to heavy metals like lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg), nickel (Ni), copper (Cu), zinc (Zn), and manganese (Mn) [48, 49].

- Several tumors do not initiate with mutations like those induced by hormonal insults. From 1940 through the 1960s, diethylstilbestrol (DES), a synthetic estrogen, was given to pregnant women to prevent pregnancy complications and losses. But such exposure resulted in increased incidence of cancer in the daughters born to DES-exposed mothers [50, 51]. Interestingly such cancers induced by environmental carcinogens/endocrine insults have no associated somatic mutations in oncogenes or tumor suppressors. The introduction of genetic instability results in cancer. Barrett et al. [52] discussed that some carcinogens can induce neoplastic transformation in the absence of somatic mutation. Boyd et al. [53] found no evidence of mutations in K-ras or H-ras (common proto-oncogenes) or in the Wilms tumor (WT1) suppressor gene or in estrogen receptor. There was no mutation in p53 but it showed increased expression. Sonnenshein et al. [54] discussed that both animal data and clinical studies suggest that cancer arises due to early developmental insults to non-mutagenic xenoestrogens including Bisphenol A. Using these models, Soto and Sonnenschein [55] put forth the TOFT theory and we recently used a similar model to further show that the tissue-resident stem cells dysfunctions initiate cancer (discussed ahead).
- Embryonic stem cells (with no mutations) form teratocarcinoma when transplanted into a normal tissue. However, these stem cells behave normally and functionally integrate when transplanted into a developing embryo. Similarly, the microenvironment can help reverse the fate of cancer cells to a normal state which is against the SMT theory that suggests the irreversible nature of cancer. Reports are available showing that cancerous cells reverse when placed in a normal 'microenvironment' [56–59].
- Variation in cancer risk amongst various tissues in the human body is poorly explained by SMT. Life-time risks for developing cancer varies in different organs being 6.9% for lungs, 1.08% for thyroid, 0.6% for brain and nervous system, 0.003% for pelvic bone, and 0.00072% for laryngeal cartilage. SMT also fails to explain why different parts of alimentary canal have varying risk of developing cancer which differs by as much as a factor of 24 [esophagus (0.51%), large intestine (4.82%), small intestine (0.20%), and stomach (0.86%)] [60]. The role of environmental factors in initiating cancers is not accounted for by SMT. Clinical studies suggest that the risk of cancer is increased by hormone therapy (several cancer types) [61, 62], shift work that involves circadian disruption (breast cancer) [63–66], and exposure to non-ionizing electromagnetic radiation (childhood leukemia) [67, 68].

In addition to SMT, several other theories exist to explain cancer initiation including the tissue organization field theory (TOFT) and cancer stem cells (CSCs). Ideally, any concept that can explain various SMT-related paradoxes should be acknowledged.

Tissue Organization Field Theory

The Tissue Organization Field Theory (TOFT) was popularized by Soto and Sonnenschein [46, 49]. The central idea was that the initial cause of cancer is not genetic mutations, but disruption of tissue organization- "development gone awry". This disorganization could be due to the chemical alteration of the extra-cellular matrix by carcinogens. Compared to SMT, which is a cell-based, 'irreversible' disease driven by somatic mutations, TOFT suggested that the default state of a cell is proliferation and cancers occur at the tissue level. According to the TOFT concept, mutations are a byproduct, and thus cancer is reversible [55, 69]. TOFT posits that cancer is a tissue-based disease whereby carcinogens (directly) and mutations in the germ line (indirectly) alter the normal interactions between the diverse components of an organ, such as the stroma and its adjacent epithelium.

However, it is felt that neither acquiring mutations nor damage to the tissue alone, is sufficient to trigger carcinogenesis. Rather, Rosenfeld [70] suggested that SMT and TOFT complement each other in a single unified theory of carcinogenesis. Challenging the century-old SMT has resulted in a lot of discussion both in support [71] and against it [18, 25, 26]. Both theories face challenges. TOFT theory fails to explain differences in cancer incidence with age and among different tissues although exposed to similar levels of DNA-damaging agents [72]. Some intriguing observations that cannot be explained by both SMT and TOFT are highlighted below.

- Although small and large intestines are exposed to similar dietary carcinogens, colorectal cancer occurs more frequently than cancer in the small intestines [72].
- Many DNA-damaging agents enter the blood and reach all tissues, including the prostate and the heart. However, prostate cancer is over 100,000 times more frequently diagnosed than heart cancer [72]. This discrepancy was later explained based on differences in cell turnover in the two organs, prostate glandular epithelium has a much higher turnover than cardiomyocytes. Tobacco is a risk factor for carcinoma. Its use leads to the formation of DNA-damaging agents and lung cancer in heavy

smokers is known to occur approximately 20 times more frequently than lung cancer in non-smokers. However, the striking differences in cancer incidence among tissues exposed to similar levels of DNA-damaging agents like the lungs and larynx indicate that exposure to these agents is not the driving force of carcinogenesis. Larynx and lungs are equally exposed to tobacco smoke; however, lung cancer is much more common than larynx cancer [37]. This is because tobacco and smoke products tend to stay longer in the lungs, with a much larger exposed surface compared to the larynx. Moreover, larynx carcinoma is much more frequent in patients exposed to both tobacco and alcohol.

Because of the above-described limitations associated with SMT and TOFT theory for cancer initiation, it is worth discussing whether cancer stem cells (CSCs) are the possible driving force behind carcinogenesis.

Role of Stem Cells in Cancer Initiation, Metastasis and Recurrence

The concept that stem cells have a role in cancer initiation, metastasis, and recurrence has been known since 1937 [73]. Focus shifted to mutations in oncogenes and tumor suppressor genes in the 1970s. But later, John Dick's group revived the CSC theory by xenografting experiments and showing that $CD34^{+}CD38^{-}$ adult human acute myeloid leukemia (AML) cells can repopulate the bone marrow of mice [74]. Later Clarke's group reported $CD44^{+}CD24^{-/low}Lineage^{-}$ CSCs in breast cancer [75]. One hundred $CD44^{+}CD24^{-/low}$ $Lineage^{-}$ cells could form tumors upon transplantation in mice while 10,000 of cells with alternate phenotype failed to do the same. However, several doubts exist regarding CSCs and even today the identity and cell surface markers to isolate CSCs and quiescent stem cells in normal tissues elude the scientific community. It was initially suggested that two populations of stem cells exist in adult tissues including relatively quiescent stem cells and actively dividing progenitors [76, 77]. However, genetic lineage tracing using the tail epidermis of transgenic mice could detect only one type of progenitor cell which divided rapidly and underwent clonal expansion [78]. Stem cells in hair follicle bulge were studied by confocal microscopy of whole mount epidermis. At 2 days post-induction, few single, labeled cells were observed (1 in 600) in the basal layer, which showed clonal expansion over time. However, the group failed to detect any quiescent cell and thus concluded that only one type of progenitor cell exists which divides rapidly [78].

Based on these initial results and several other reports, Post and Clevers [79] concluded that stem cells in solid organs do not show properties like rarity, specific marker expression, quiescence, asymmetric division, and unidirectional differentiation like the hematopoietic stem cells. They suggested that quiescence may not be always necessary for stemness. Actively dividing progenitors and even differentiated cells suffice to replace lost tissue. It was suggested that stem cells in adult tissues should be defined based on their function and not their phenotype [79, 80]. Shivdasani et al. [81] discussed that adult tissues possibly lack a dedicated stem cell compartment and that stem-like progenitors arise due to dedifferentiation and reprogramming of somatic cells to overcome chronic insults/injury. Similarly, quiescent CSCs have also remained elusive and may neither be rare nor quiescent, rather they could be abundant and proliferate vigorously [82]. However the absence of evidence is not always evidence for absence! They failed to detect quiescent stem cells based on their lineage tracing studies but quiescent stem cells do exist in the G0 stage of the cell cycle in multiple tissues (discussed ahead). These quiescent stem cells in G0 stage will never get picked up by lineage tracing which can only track cycling cell populations. Sometimes the glamour of technology makes scientists forget basic scientific principles and this leads to misconceptions in the field.

Tomasetti and Vogelstein [60], with their out-of-box thinking, were intrigued by the fact that the lifetime risk of being diagnosed with cancer varies for different organs, being 6.9% for the lung, 1.08% for the thyroid, 0.6% for the brain and the rest of the nervous system, 0.003% for pelvic bone and 0.00072% for laryngeal cartilage [www.seer.cancer.gov]. They suggested that the lifetime risk of cancer was strongly correlated with the divisions of normal self-renewing stem cells that maintain tissue homeostasis. Only one-third of the variation in cancer risk was attributed to environmental factors such as smoking, alcohol use, ultraviolet light, human papilloma virus or inherited predispositions. But such exposures could not explain why cancer risk in tissues within the alimentary tract differ by as much as a factor of 24 [esophagus (0.51%), large intestine (4.82%), small intestine (0.20%), and stomach (0.86%)]. Moreover, cancers of the small intestinal epithelium are three times less common than brain tumors, even though small intestinal epithelial cells are exposed to much higher levels of environmental carcinogens/ mutagens than are cells within the brain, which is protected by the blood–brain barrier. Based on this, they proposed that rather than extrinsic factors like environmental factors or inherited predispositions, intrinsic total stem cell turnover, is closely related to a lifetime risk of developing a particular cancer. Random mutations arising during DNA replication in normal, non-cancerous stem cells are

the major cause of cancer initiation [60]. The group concluded that Bad Luck mutations due to DNA replication errors during stem cell mitosis, play a predominant role in cancer initiation. Two examples described by them to support their views are mentioned below.

- Melanocytes and basal cells of the skin epidermis are both exposed to the same carcinogens (UV light) at a similar dose. However, melanoma is less common compared to basal cell carcinoma because basal cells undergo a higher number of cell divisions [60].
- Cells in duodenum and small intestine divide 1/3 and 2/3 times less frequently compared to large intestine and this explains why colorectal cancer of the large intestine is more common [83, 84]. Interestingly in mice, the small intestine undergoes more stem cell divisions than the large intestine [85, 86]. This explains why mice with inherited APC mutations show a higher incidence of cancer in the small intestine compared to humans with FAP mutations who are approximately 30 times more likely to develop CRC [60].

This article [60] directly challenged the SMT theory and attracted more than 25 commentaries on Pubmed. The concept that the number of normal cell divisions dictates cancer risk in many organs was further confirmed by studies in mice [87]. Later, they studied the incidence of 17 cancer types in 69 countries and observed a strong correlation (median = 0.80) between cancer incidence and normal stem cell divisions in all the countries regardless of their environment. They showed that 66% of cancer-causing genetic mutations arise from the "bad luck" of a healthy, dividing cell making a random mistake when it copies its DNA during mitosis [88]. A major criticism of their view was their failure to acknowledge the role of external environmental and genetic factors in cancer initiation. The concept of Bad Luck mutations put forth by Tomasetti and Vogelstein [60, 88] was challenged by Wu et al. [89] who stated that besides stem cell turnover in the body, extrinsic influences due to the environment, bad habits, poor diet, improper lifestyle also have a significant contribution towards cancer initiation. They provided data suggesting that intrinsic factors contribute to < 10–30% of the risk of developing common cancers and the risk is heavily influenced by extrinsic factors. Examples to support the role of extrinsic factors in cancer are listed below.

- Large international geographical variations exist in the incidences of breast and prostate cancer (fivefold for breast cancer, 25-fold for prostate cancer) [90], and immigrants moving from countries with lower cancer incidence to countries with higher cancer rates soon acquire the higher risk of the new country [91, 92].
- Colorectal cancer is another high-incidence cancer that is widely considered to be an 'environmental' disease [93], with an estimated 75% or more colorectal cancer risk attributable to diet [94].
- Environmental risk factors for several cancers have been identified. For example, 65–86% of melanoma risk is ascribed to sun exposure [95]. ~ 90% of non-melanoma basal and squamous skin cancers are attributed to UV [96]. At least 75% of esophageal cancer, or head and neck cancer are caused by tobacco and alcohol [97, 98].
- Certain pathogens dramatically increase the risk of cancers. For instance, the human papillomavirus may cause ~ 90% cases of cervical cancer [99], ~ 90% cases of anal cancer [100] and ~ 70% of oropharyngeal cancer [101]; Hepatitis B and Hepatitis C virus may account for ~ 80% cases of hepatocellular carcinoma [102]; and Helicobacter pylori may be responsible for 65–80% of gastric cancer [103].

These examples provided direct evidence that environmental factors play important roles in cancer incidence and they are modifiable through lifestyle changes and/or vaccinations. It was suggested that both Bad Luck and 'punishment' because of bad habits may together result in cancer initiation [104].

After the above-described correlation between cancer incidence and stem cell divisions was reported [60, 88], Lopez-Lazaro [37, 72] compared the number of gene mutations and cancer risk across tissues. He analyzed the whole genome sequencing information from 22,086 cancer samples and incidence data from the largest cancer registry in each continent to study the relationship between the number of gene mutations and the risk of cancer across 33 tissue types. Surprisingly, the results showed a weak positive correlation (mean = 0.14) between the two parameters. The correlation became stronger (mean = 0.50) when gender-related cancers were excluded. Results also showed that 1,003 samples from 29 cancer types had zero mutations. These results provided further support to the view that cancer etiology can be better explained by the accumulation of stem cell divisions than by the accumulation of gene mutations. Various concerns raised by Lopez-Lazaro [37, 72] regarding the SMT and TOFT theories for cancer initiation were discussed above. He proposed the Stem Cell Division Theory of Cancer (STDTC) [37, 72] wherein both stem cell proliferation and changes in their microenvironment have a role in cancer initiation. It was proposed that (i) cancer initiates from normal stem cells due to the accumulation of DNA changes during cell divisions (ii) accumulation of DNA changes in stem cells results in the generation of cancer stem cells (CSCs), which are responsible for tumor formation (iii) metastasis occurs when CSCs leave their natural tissue and form tumors in other locations (iv) cancer formation requires the interaction of

stem cells with accumulated DNA changes and variations in their microenvironment. Lopez-Lazaro [72] also discussed the role of stem cells in explaining cancers of unknown primary sites. STDTC theory of cancer initiation is supported by several observations including.

- Increased cancer incidence observed in aged people
- Increased risk of developing esophageal cancer with drinking very hot beverages
- Cancers caused due to long-term exposure to non-ionizing radiation
- Circadian disruption of electrical lighting increases the risk of developing breast cancer.

STDTC theory for cancer initiation provided strong support to the concept that most human cancers arise due to the accumulation of mutations in the normal stem cells which eventually convert into CSCs. CSCs play a crucial role in tumor growth, metastasis, and resistance to treatment; however, the origin and identity of CSCs lack consensus and whether the CSCs model applies to few or many cancers. It was also suggested that stem cells in normal tissues that regenerate after an acute injury like partial pancreatectomy or hepatectomy and in cancerous tissues that initiate cancer possibly do not exist. Rather they form by de-differentiation and in vivo reprogramming of somatic cells/progenitors [81, 105]. More than 90% of the solid tumors arise in epithelial tissues. To become malignant, epithelial cells undergo a transition to a mesenchymal state by a process termed epithelial-to-mesenchymal transition (EMT). Upon reaching a secondary site, cells undergo the opposite transition, the mesenchymal-to-epithelial transition (MET). Weinberg's group [2] was the first to show that EMT in the epithelial cells in solid tissues is a source of MSCs as well as CSCs with an increased ability to form spheres by clonal expansion and this concept exists even today [106, 107].

Source of Cancer Stem Cells: Dedifferentiation of Somatic Cells or Expansion of Tissue-resident Stem Cells?

Trosko [108] refuted the concept of dedifferentiation and reprogramming of somatic cells during EMT as the mechanism for the appearance of CSCs based on his 'Kiss of Death' assay. According to him, any human organ biopsy comprises large numbers of terminally differentiated cells, progenitors, and tissue-specific adult stem cells. When dissociated cells from a biopsy are placed on an irradiated feeder support, terminally differentiated cells remain floating and die, and progenitor cells attach but eventually die. After a week, a few small clones of proliferating cells are invariably observed which are the stem cells and express nuclear OCT-4. Thus, he questioned the concept of dedifferentiation and reprogramming of somatic cells into CSCs in favor of endogenous, tissue-resident stem cells. Based on similar reasoning, he also argued that induced pluripotent stem (iPS) cells reflect expansion of normal, tissue-resident fibroblast stem cells rather than dedifferentiation and reprogramming of somatic cells in vitro. Another group [109] has also reported that skin fibroblast cells fail to produce iPS cells if a subpopulation of pluripotent multilineage-differentiating stress-enduring (MUSE) cells is removed from the primary culture, thereby suggesting that iPS cells possibly reflect the expansion of a subpopulation of pluripotent stem cells (MUSE cells) in vitro rather than dedifferentiation and reprogramming of mature somatic fibroblasts. It is still not resolved whether the iPS cells arise from pre-existing stem cells or the reprogramming of differentiated cells. Similarly, the concept of dedifferentiation and reprogramming of mature somatic cells to give rise to CSCs during the epithelial-mesenchymal transition of epithelial cells in solid organs challenges the basic paradigm of development biology regarding plasticity that a cell enters a point of no return once it initiates differentiation [110].

Yamanaka's group has addressed the concerns regarding the origin of iPS cells (whether it is by reprogramming mature somatic cells or by the expansion of a subpopulation of tissue-resident stem cells in vitro) since their initial publications which fetched them the Nobel Prize in 2012 [111, 112]. Only a small portion of fibroblasts (< 1%) get reprogrammed into iPS cells when subjected to the reprogramming cocktail. The low frequency of reprogramming to give rise to the iPS cells could be due to the co-existence of rare tissue stem/progenitor cells in the fibroblast cultures. Almost 0.067% of mouse skin cells are stem cells [113–115]. But Yamanaka's group proposed that similar to the ability of oocytes/zygotes to spontaneously reprogram a somatic cell to a pluripotent state, the introduction of four transcription factors could reprogram skin fibroblasts to a pluripotent state. Okita and Yamanaka in 2010 [116], discussed this issue further and supported reprogramming over the expansion of tissue-resident stem cells by giving several examples. Besides skin fibroblasts, liver hepatocytes and gastric epithelial cells [117], terminally differentiated mature B lymphocytes [118], and pancreatic β cells [119] also successfully reprogram to generate iPS cells. With time, the efficiency of reprogramming was improved by up to 5%. Yamanaka's group again addressed the possibility that a rare population of stem/progenitor cells co-existing in somatic cell culture could give rise to iPS cells in 2013 [120]. Interestingly, an earlier study in mice [121] showed that ectopic expression of Oct-3/4 in the mouse stomach and intestine resulted in dysplastic growths in epithelial tissues and expansion of epithelial progenitor and/or stem cells. Conversely, *Oct-4* expression in already differentiated cells of the intestine or

hair follicle did not affect the cellular phenotype, and ectopic expression of *Oct-4* in differentiated fibroblasts exerted adverse effects on cell proliferation.

Tissue-resident, pluripotent stem cells are very small in size and could pass along with the mature cells even after sorting by a process termed 'emperipolesis' as discussed for VSELs [122] similar to megakaryocytes carry RBCs and macrophages carry lymphocytes and plasma cells [123]. While deriving iPS from blood cells, usually mononuclear cells (MNCs) are infected to get reprogrammed [124, 125] and there is no clarity whether blood cells are getting reprogrammed or the VSELs that exist as a sub-population amongst the MNCs [122, 126] start expanding in culture. Similarly, the starting culture used for reprogramming hepatocytes by Yamanaka's group [117] was indeed heterogeneous [127] and thus it is possible that initial cultures had a sub-group of VSELs amongst them. Lineage tracing experiments revealed that iPS cells expressed albumin and thus Yamanaka's group argued that differentiated hepatocytes were reprogrammed to give rise to the iPS cells [117]. However, they reported both GFP positive and negative colonies in culture and it is likely that pluripotent VSELs in the initial cultures, increased in numbers and differentiated into both β-gal positive and negative cell types. We have earlier reported that VSELs exist amongst β islets in mouse pancreas [128] (Fig S1) and thus it is not clear which cells grew in the culture as colonies in the study reported by Stadtfeld et al. [119].

It is likely, that iPS cells may not be the result of dedifferentiation and reprogramming of mature somatic cells in vitro but since the initial culture is heterogenous, it is likely that pre-existing stem cells expand in vitro. Similarly, cancer initiation may not necessarily be due to dedifferentiation and reprogramming of epithelial cells in vitro as widely suggested, because if this was true, iPS cells should form carcinomas or sarcomas (which normally arise from the epithelial cells) and not teratomas upon transplantation. Teratomas comprising cells of all three lineages can only arise from a pluripotent cell and not from differentiated epithelial cells. Trosko's work was based on two markers including OCT-4A for stem cells and connexin (GJIC- gap junctional intercellular communication) which was suggestive of differentiation. He reported the presence of two types of CSCs including OCT-4A-expressing cells (not expressing GJIC) and those derived from these cells expressing GJIC (suggesting early differentiation but blocked terminal differentiation).

As discussed above, Hans Clevers and his group failed to detect label-retaining, quiescent stem cells in normal and cancerous tissues, and their research is now focused on epithelial leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) positive stem cells which are dependent on Wnt signaling [129–131]. In vivo lineage tracing studies reported by them have revealed that actively proliferating, yet long-lived Lgr5 + cells contribute to the renewal of gut epithelium, and similar stem cells are reported in a variety of epithelial organs including hair follicle, small intestine, colon, endometrium, pancreas, lungs, and liver [132, 133]. A single Lgr5 + intestinal stem cell can initiate 3D organoid formation [134]. These clonally expanding Lgr5 + cells from multiple organs readily form organoids in culture [135, 136] and are considered ideal predictive models for translational oncology [137]. Based on their ability to divide and undergo clonal expansion, Lgr5 + cells are most likely early, transit-amplifying cells or progenitors that form organoids in vitro but we suggest that surely, they are not cells of origin of cancer which are expected to exhibit the properties of asymmetrical cell division and quiescence. Use of 3D organoids positive for Lgr5, for drug screening assays, by pharma companies may not lead to the development of an effective cancer cure as these drugs will fail to target quiescent stem cells. Melo et al. [138] reported that selective Lgr5 + cell ablation restricted primary tumor growth, but did not result in tumor regression. They suggested that tumors are maintained by proliferative Lgr5 − cells that continuously replenish the Lgr5 + CSC pool. This is because the quiescent CSCs (unlike clonally expanding Lgr5 + cells), will survive both chemo- and radiotherapy and invariably result in recurrence. Similarly, immunotherapy does not target the quiescent CSCs. Since the stem cells comprise < 0.1% of total cells in any tissue, OMICS studies on intact tissues fail to provide insight into the tissue-resident stem cells. We postulate that tissue-resident, pluripotent, very small embryonic-like stem cells (VSELs) have a crucial role to play in both the normal turnover of cells in multiple organs and during cancer initiation. Their presence in adult tissues challenges the concept of the formation of CSCs by dedifferentiation/reprogramming of epithelial cells during in epithelial-mesenchymal transition in solid tumors.

However, a huge amount of published data exists to support the concept of dedifferentiation and reprogramming of somatic cells to form CSCs [139]. Complex genetically modified mice models are used by various investigators to produce evidence in support of the dedifferentiation of somatic cells into CSCs. The basic cause of the existing misperceptions in the field are because the scientific community fails to acknowledge the presence of pluripotent VSELs in adult tissues. Two studies [140, 141], which Walcher's group cited [139], provide evidence to support the dedifferentiation of somatic cells as a source of CSCs are discussed in the supplementary section (please refer to pages 8-14 of the supplementary section). A careful analysis of the results of these studies suggests that it is also possible that tissue-resident VSELs could initiate cancer but both studies remain mute on this aspect since the authors do not acknowledge the presence of VSELs in adult tissues. The field of cancer biology requires a huge kerfuffle as suggested by Baker [18] as this

would open new lines of research by spurring critical thinking. A complete paradigm shift in favor of tissue-resident, quiescent, and pluripotent VSELs is required to reduce the global cancer burden. The aim of the current review is not to comment on the regenerative potential of iPS cells which hold great promise but to discuss their origin; only to put cancer stem cell biology in proper context.

Quiescent, Tissue-resident, Pluripotent VSELs Transform Into CSCs

Pluripotent, very small embryonic-like stem cells (VSELs) with nuclear OCT-4A, reported for the first time by Ratajczak's group, reside in small numbers in all adult tissues (amongst the epithelial cells in solid tissues) and function along with tissue-specific progenitors to maintain life-long homeostasis [142, 143]. Besides maintaining life-long tissue homeostasis, VSEL dysfunction results in various pathologies including cancer and age-related pathologies [144]. However, VSELs have eluded the scientific community for decades because of their small size and scarce nature [145]. Despite the controversy regarding their presence that surfaced a decade ago [146], the presence of VSELs has now been confirmed by more than 50 independent groups worldwide and robust protocols are published to enrich them from the bone marrow/blood or any solid tissue [142, 147–150]. VSELs are observed amongst the endometrial epithelial cells lining the lumen and the glands, testicular seminiferous tubules (Fig. 1), and also in the ovary surface epithelium (Fig S2).

VSELs are quiescent, exist in the G0 stage of the cell cycle [150], survive whole-body radiation [151] and treatment with busulphan and cyclophosphamide [152, 153] in mice studies. Kurkure et al. [154] showed the presence of VSELs in the testicular biopsies of young, azoospermic survivors of childhood cancer suggesting the quiescent nature and ability of VSELs to survive oncotherapy. Research is ongoing to manipulate the VSELs, in the ovaries and testes that survive oncotherapy, to serve as a source of gametes to achieve biological parenthood [155]. Mice studies have shown that early-life endocrine insults alter the epigenetic status of VSELs in the endometrium and testes [156–159]. As a result, VSELs transform into CSCs, come out of their quiescence, enter the cell cycle and undergo excessive self-renewal but their further differentiation is blocked. This disbalance between excessive self-renewal (proliferation) of VSELs and their further differentiation results in cancer (Fig. 2). A similar increase in VSELs numbers was also reported in aged mice ovaries with bilateral cysts [160]. Increased numbers of VSELs have also been reported in human ovarian cancer biopsies by Virant-Klun and co-workers [161–163]. Ratajczak's group was the first to propose that VSELs are the 'embryonic remnants' that transform into CSCs and the misappropriate differentiation initiates cancer [164, 165]. OCT-4 and FSHR, reported in multiple cancer biopsies are also expressed on VSELs and this suggests that the cell-of-origin of CSCs is VSELs and not epithelial cells. Evidence demonstrating the role of VSELs in initiating cancer was recently discussed [11, 166]. However, these are early results based on mice and few human studies. More data needs to emerge on different types of cancers and on different aspects but for this global interest needs to be generated in VSELs.

The recently reported findings of Bhartiya's group, provide promising evidence for cancer initiation from tissue-based VSELs that transition into CSCs due to epigenetic changes [156–159] and thus provide an altogether novel view on cancer initiation, metastasis, and recurrence. Cancer is indeed a stem cell disease as suggested by other groups as well and various associated key findings are discussed below.

- Two populations of stem cells exist in adult tissues, including quiescent, pluripotent VSELs and actively dividing progenitors (adult stem cells), which are lineage-restricted and tissue-specific [Fig. 3, Fig S3]. These two populations of stem cells work together to maintain life-long tissue homeostasis. Rather than dedifferentiating and reprogramming somatic cells into normal and cancer stem cells [2], tissue-resident VSELs participate in maintaining life-long homeostasis, regular turnover of cells in various organs, regeneration after chronic injury, and undergo epigenetic changes to convert into CSCs and initiate cancer. Being quiescent, tissue-resident VSELs (exist in the G0 stage of the cell cycle) have not been detected by the lineage-tracing studies, which mark only dividing cell populations. A better method to track quiescent stem cells is to flow-sort GFP-tagged quiescent VSELs and study their functional potential upon transplantation [167].
- VSELs are scarce and of small size, inadvertently get discarded while processing cells for various studies, remain elusive, and are not yet widely accepted [145]. VSELs fail to be detected by flow cytometry [146] and multiple single-cell RNA-Seq studies. Underlying reasons for the same including their small size and scarce nature have been discussed [143, 145, 168]. Robust protocols are now available to study them by flow cytometry [147–149] and several groups have confirmed their presence [142].
- VSELs exist in the basal region of epithelial cells in the ovarian surface epithelium [149, 169] (Fig S2) and the luminal and glandular endometrial epithelium [148] (Fig. 1A). Their numbers increase upon FSH treatment in testicular seminiferous tubules (Fig. 1B-C) [170]. A similar presence of VSELs is expected in all other epithelial organs and has been reported [126, 171]. Epige-

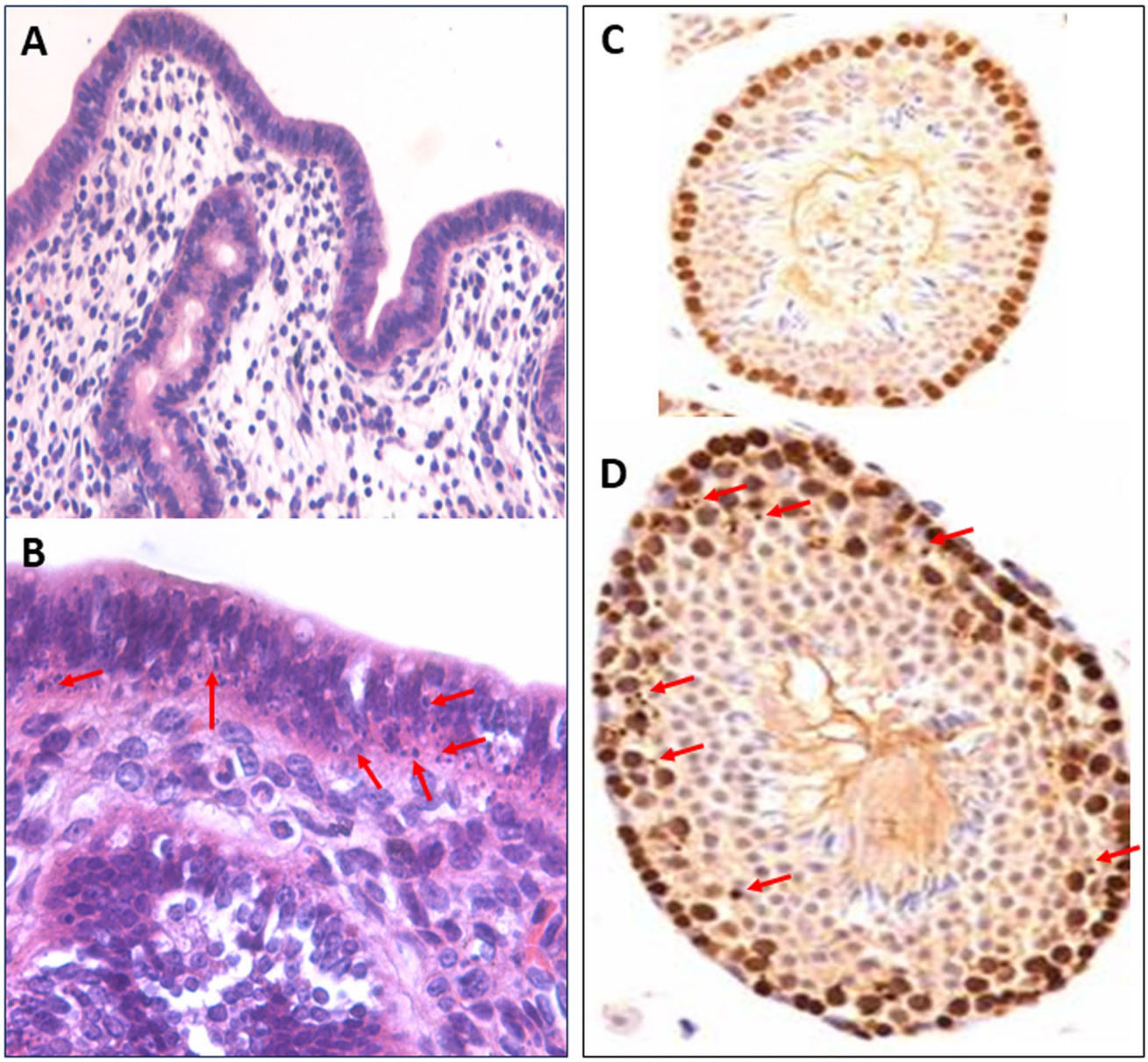

Fig. 1 Seeing is believing. Note the presence of small, spore-like, tissue-resident spherical, putative VSELs (red arrows) in mouse uterine and testicular sections. VSELs are not easily detected in normal tissues since they are scarce, but become evident upon experimental manipulations. **A.** Mouse uterine section after bilateral ovariectomy on D30 and then treated with estradiol and progesterone to result in secretory phase endometrium on day of sacrifice (DOI:10.1007/s12015-021-10279-8B). **B**. Mouse pups were treated with estradiol (20 µg/pup/day on days 3–7) followed by similar treatment as shown in **A**.. VSELs were detected (red arrow) amongst the epithelial cells in mouse endometrial sections. This image challenges the concept of CSCs arising by epithelial-mesenchymal transition for cancer initiation [2, 106, 107]. The stem cells increased in numbers and were clearly evident in adult endometrium upon neonatal exposure to endocrine disruption. VSELs in mouse uterine endometrium have been characterized in detail [148], evidence to support their differentiation into epithelial cells is published [167], their increased numbers during the diestrus state of the estrus cycle under physiological conditions [148] and their activation upon inflicting mechanical injury [167] has been delineated. VSELs role in initiating endometrial cancer has been reported [156, 157]. Proliferating cell nuclear antigen (PCNA) expression in the mouse testicular sections from **C** normal and **D** after 15 h of treatment with pregnant mare serum gonadotropin [170] (PMSG, FSH analog, 10 IU). Note the presence of PCNA-positive germ cells along the basal region. PMSG treatment resulted in the presence of PCNA-positive, small-sized VSELs (red arrow) along with increased numbers of germ cells (2–3 layers compared to 1 layer in untreated control). VSELs express FSHR and thus increased in numbers upon FSH treatment and increased PCNA expression was observed [170]. PCNA expression is intense in small-sized VSELs and in small-sized germ cells in the outer layer and staining intensity shifts from the nucleus into the cytoplasm and gradually disappears as the stem cells undergo further differentiation into spermatogonial cells. Similar presence of VSELs in mouse pancreatic islets and ovary surface epithelial cells is shown in Fig S1-2

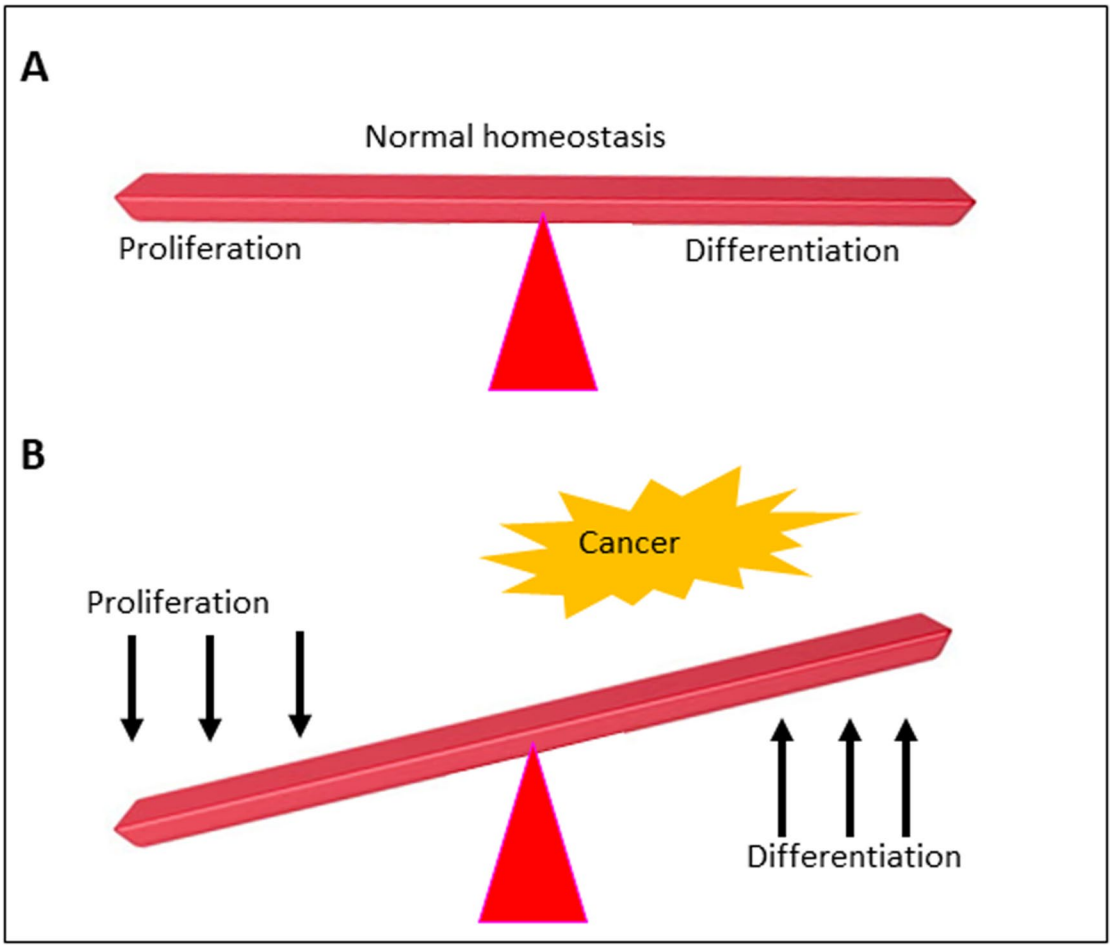

Fig. 2 Cancer is a stem cell disease. **A**. Stem cells function in a subtle manner to maintain a balance between proliferation and differentiation resulting in homeostasis under normal conditions. **B.** Stem cell dysfunctions result in a disbalance between proliferation and differentiation. Various environmental insults result in epigenetic changes in the VSELs and as a result, the normally quiescent VSELs in G0 stage of cell cycle, enter cell cycle as CSCs and undergo excessive self-renewal to initiate cancer

netic changes in VSELs result in their increased numbers and transition into CSCs. In addition, the tumor stromal compartment comprising MSCs, pericytes, endothelial cells, and nerve fibers possibly differentiates from the pluripotent VSELs, as discussed recently [11].

- Small-sized VSELs undergo asymmetrical cell divisions (ACD) whereby they self-renew and give rise to the slightly bigger progenitor of distinct fate based on their location. The progenitors in turn undergo symmetrical cell division (SCD) and clonal expansion in multiple tissues [172] before eventually differentiating into tissue-specific cell types (Fig S2). Differential expressions of NUMB and OCT-4 have been reported in cells undergoing ACD [173] (Fig S4A-B). Both ACD and SCD along with clonal expansion have also been delineated in vivo in mouse uterine sections (Fig S5).
- VSELs express receptors for gonadal and gonadotropin hormones [147–149, 174–177], and thus respond to hormones in circulation and are directly vulnerable to endocrine insults. This characteristic property of VSELs provides the much sought-after connection between extrinsic influences like endocrine-disrupting chemicals and tissue-specific cell turnover, closely associated with cancer initiation [60]. Being virtually immortal, only VSELs have the potential to carry endocrine insults during early development to adult life and result in various pathologies including cancer. Other somatic cells (that also get exposed to developmental endocrine insults) have a limited life span and are regularly replaced by the progenitors arising from the VSELs. Based on their numbers in different organs [176], cell turnover may vary in different organs and hence the incidence of cancer.
- Being pluripotent, VSELs have 'open' chromatin, minimal 5-methyl cytosine expression and exhibit biallelic erasure of genomic imprints. The biallelic expression of H19 and silenced IGF2 on both alleles explains the quies-

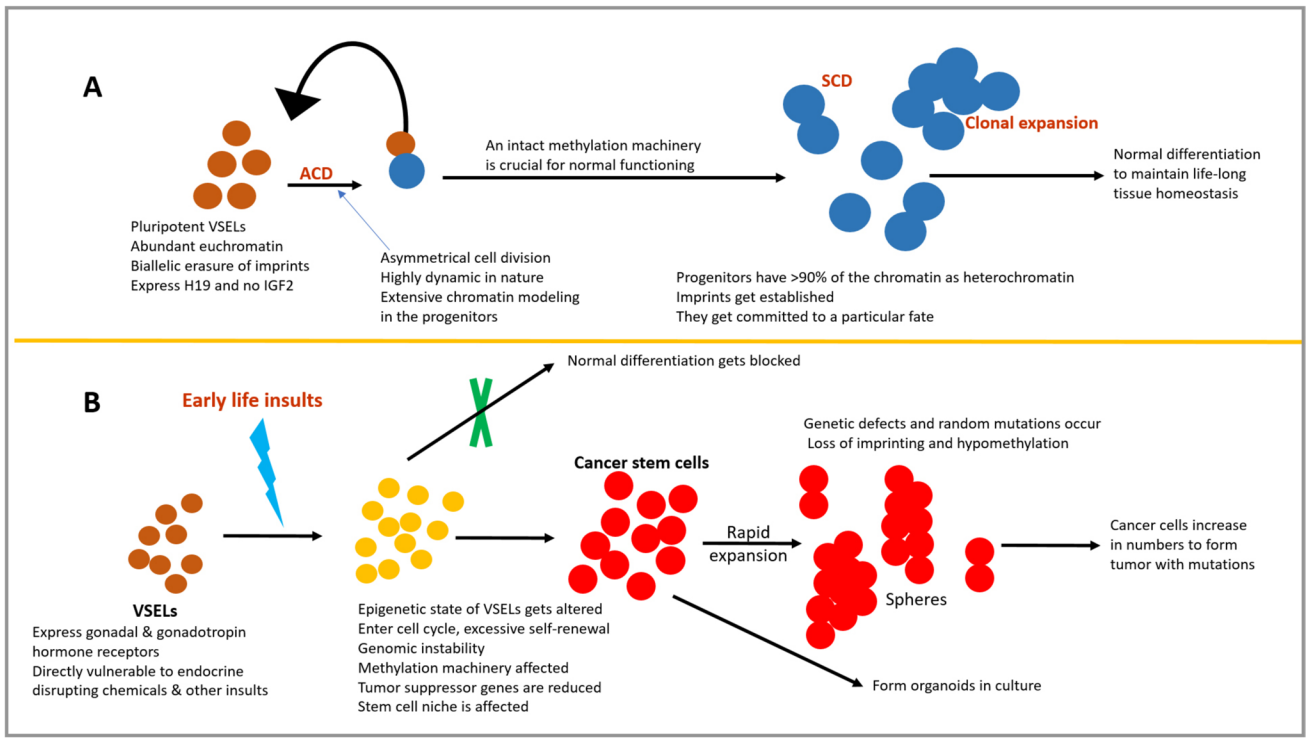

Fig. 3 Stem cell biology in normal tissues and during cancer. **A**. Upper panel shows how tissue-resident, pluripotent VSELs function under normal conditions to maintain life-long tissue homeostasis. VSELs normally remain quiescent and in G0 stage of cell cycle. They occasionally undergo asymmetrical cell division (ACD) to give rise to two cells of different size and fate. The smaller cell enables self-renewal of VSEL while the bigger one is a progenitor cell that divides and expands in numbers by undergoing symmetrical cell divisions (SCD) and clonal expansion (sphere formation) before initiating differentiation into tissue-specific cell types. **B.** VSELs are directly vulnerable to environmental insults throughout life including early-life exposures. This is because VSELs express receptors for gonadal and gonadotropin hormones and most of endocrine-disrupting chemicals are estrogenic in nature. Being virtually immortal, they carry the insults to adult life and initiate pathologies including cancer. VSELs undergo epigenetic changes and transform into cancer stem cells (CSCs). CSCs undergo rapid expansion in numbers, exhibit genomic instability and hypomethylation, loss of imprinting (LOI), and altered DNA damage repair (DDR) and mismatch repair (MMR) axis. Random mutations occur as a consequence of the rapid proliferation of CSCs; mutations do not initiate cancer. The functionally defective and quiescent VSELs are not targeted by the existing therapies. They are responsible for recurrence and result in the appearance of more novel mutations upon therapy. This explains why targeting mutations during personalized medicine does not help. Similarly, drug discovery using organoids in vitro will only target the actively dividing cancer cells. Need to target VSELs to Win the War against Cancer and to achieve the Cancer Moonshot

cence of VSELs and has been reported for bone marrow VSELs [178, 179]. Every time a VSEL undergoes ACD to self-renew and give rise to a lineage-restricted and tissue-committed progenitor, > 90% of the chromatin becomes inactivated ['permissive' euchromatin is converted into heterochromatin]. Moreover, genomic imprints also get established with monoallelic expression of H19 and IGF2 in the progenitors. These events of DNA methylation and genomic imprinting require intact machinery to ensure normal methylation. DNA Methyltransferases (DNMTs) carry out methylation at dinucleotide CpG sites, but only three of them possess methyltransferase activity. DNMT1 is a maintenance methyltransferase (maintains the existing methylation patterns following DNA replication and catalyzes the addition of 5-mC to the newly replicated strand during the S phase). At the same time, DNMT3A and DNMT3B are responsible for de novo methylation along with DNMT3L (does not possess DNA methyltransferase activity, but it is required for DNMT3a and DNMT3b functions). Hypomethylation and hypermethylation cause either the expression or inhibition of genes, and there is a tight balance between the regulation of the activation or repression of genes during normal conversion of pluripotent to a committed state and further cellular differentiation. DNA methylation, catalyzed by the DNMTs, plays an important role in maintaining genome stability. Aberrant expression of DNMTs and disruption of DNA methylation patterns are closely associated with many forms of cancer [180].

- Epigenetic changes are common in cancer. Global DNA hypomethylation is a ubiquitous feature and critical determinant of multiple cancers. Global hypomethylation is observed very early during cancer initiation and progression and is associated with loss of imprinting (LOI)

of imprinted genes and confers immortalization on cells. In cancer, LOI reactivates normally silent alleles and/or silences the normal active allele of a tumor suppressor gene. Imprinted IGF2/H19 loci are commonly affected in several childhood tumors such as Beckwith-Wiedemann syndrome (BWS), Wilms' tumor, hepatoblastoma, and rhabdomyosarcoma. The LOI of IGF2 has been reported in adult tumors, including prostate, breast, lung, colon, bladder, liver, ovarian, and cervical cancers. It has also been reported in blood malignancies [181]. LOI occurs not only in cancer cells but also in normal adjacent tissues and lymphocytes, suggesting that it is an early epigenetic defect that could predispose patients to tumor development.

- The mechanisms underlying aberrant LOI noted in several cancers are not yet understood. We suggest that this occurs because of dysfunctional VSELs with defective methylation machinery. The dysfunctional VSELs (with bilaterally erased imprints) undergo excessive self-renewal, but since the methylation machinery is defective (as discussed above), the expanding cells will show hypomethylation and LOI.
- In contrast to genetic mutations, epigenetic changes are more reversible. It is intriguing to note that this imprinted gene loci (IGF2/H19) is crucial for VSEL quiescence. Under normal conditions, IGF2 is minimally expressed, whereas increased H19 confers quiescence [178, 179]. Holm et al. [182] reported the spontaneous immortalization of imprint-free mouse embryonic fibroblasts, and mice derived from these fibroblasts (immortalized imprint-free fibroblasts) showed multiple tumors. This contrasts with the inability to induce tumors and immortalize human epithelial cells by artificial ectopic overexpression of oncogenes, as per the SMT theory for cancer initiation.
- **Available evidence for cancer initiation from VSELs**: It is well known that several pathologies including cancer initiate due to early-life extrinsic insults like exposure to endocrine disrupting chemicals. However, the underlying mechanisms that initiate these pathologies are not yet understood despite global efforts and OMICS studies. Bhartiya's group has reported that tissue-resident VSELs were affected by neonatal exposure of mouse pups to estradiol as well as diethylstilbestrol in mouse testis and uterus resulting in their increased expansion (self-renewal) while their further differentiation was blocked. VSELs entered the cell cycle, and a loss of 5-methyl cytosine was noted compared to control 100-day-old mouse tissues. There was a loss of imprinting at Igf2-H19 and Dlk-Meg 3 loci suggested by increased expression of Igf2 and Dlk. Further, expression of Dnmts, and Ezh2 were altered while tumor suppressor genes showed reduced expression. Defective methylation machinery associated with excessive self-renewal of CSCs possibly results in genetic changes – Bad Luck mutations in the cancer cells (Fig. 3) which are indeed a symptom (consequence) of cancer and have no role in cancer initiation. More work needs to be undertaken on different aspects using clinical samples to carry this research forward.
- Mutations do not accumulate in the VSELs (since they are quiescent). This makes our postulate distinct from the one put forth by López-Lázaro [37, 72] which suggested that the accumulation of mutations in the stem cells initiates cancer. Cancer-associated mutations occur due to errors in DNA replication during rapid, uncontrolled clonal expansion and sphere formation of CSCs (epigenetically altered VSELs) and cancer cells. Mutations do not accumulate due to errors in DNA damage in somatic cells as postulated by the SMT theory nor in the quiescent, tissue-resident VSELs. Our model provides an underlying mechanism for the 'Bad Luck' mutations described by Tomasetti and Vogelstein [60] which remain random (Fig. 3).
- In contrast to the irreversible nature of cancer-based on the SMT theory, the epigenetic changes in CSCs leading to cancer initiation render cancer 'reversible'. Various strategies could include targeting signaling pathways of CSCs [183], promoting differentiation of stem cells [184, 185], 'epigenetic diet', resveratrol [186], and improving the stem cells niche to reverse/prevent cancer [187]. The importance of the stem cell niche can be explained by studying the behavior of undifferentiated ES/iPS cells that form teratomas and also exhibit the ability to integrate into a developing embryo in different environments. Early detection of stem cell dysfunctions leading to cancer initiation as well as recurrence and pushing CSCs (undergoing excessive self-renewal) back to VSELs (in the G0 stage of the cell cycle) with normal functions has the potential to provide a universal cure for all types of cancer. Targeting mutations, immunotherapy or using organoids for developing newer druggable targets may not help since the real culprits (dysfunctional CSCs) will remain unaffected and will always result in recurrence.
- Blokzijl et al. [188] reported tissue-specific mutation accumulation in human adult stem cells isolated from the small intestine, colon and liver. A careful analysis of their methods shows that they worked on actively dividing progenitors. Similarly, clonally expanding spheres (organoids) in culture have been studied for mutations [189]. Results of these studies support our model (Fig. 3) but we also suggest a crucial role of tissue-specific VSELs in initiating cancer, besides the genetic changes.
- Cancer has also been discussed as a metabolic disease [190]. This concept also questions the genetic origin of cancer. We suggest that both genetic mutations and mito-

chondrial changes occur in the CSCs and cancer cells and are possibly a consequence of cancer.

- Increased OCT-4A levels were reported in the peripheral blood of 500 clinical subjects affected by 25 different types of cancer [191]. Studying VSELs using a panel of markers is an excellent approach to monitor cancer absence, presence or recurrence by a simple, non-invasive blood test [11]. Recent proof-of-concept publications [192, 193] have reported successful reversal of pathologies including cancer by treating with XAR (a nano-formulation of resveratrol, epigenetic regulator) and by transplanting mesenchymal stromal cells (provides paracrine support and improves the niche). Results suggest that cancer is a stem cell disease and can be reversed (if caught early) by normalizing stem cell functions. Similarly, several reports are available where Resveratrol [194] and MSCs [195] have been used for therapeutic purposes.

Table 1 shows various paradoxes associated with SMT and other theories related to cancer initiation, and how they could be explained by the concept that epigenetically altered VSELs initiate cancer.

Table 1 Inconsistencies associated with SMT and other theories for cancer initiation are explained by VSELs being the cell-of-origin for cancer

Inconsistencies with SMT and other theories of cancer initiation	Explanation provided based on VSEL biology
How can a mutation due to DNA damage occur in a differentiated epithelial cell that cannot divide and is on the path to senescence? MSCs differentiate into multiple cell types like endothelial cells, cancer-associated fibroblasts, and nerve cells in a tumor Dedifferentiation & reprogramming of epithelial cells result in pluripotent CSCs by undergoing EMT [2, 5, 6]	Mutations do not occur in differentiated, senescent epithelial cells but in early epithelial progenitors that arise by asymmetrical cell divisions (ACD) of the immortal VSELs that reside in few numbers amongst the epithelial cells (Fig. 1). VSELs are the most primitive & pluripotent stem cells, developmentally linked to PGCs with nuclear OCT-4A that differentiate into MSCs with cytoplasmic OCT-4. Similarly, VSELs being pluripotent, have the potential to differentiate into endothelial cells, cancer-associated fibroblasts, and nerve cells.
Cancer cells display a wide variety of somatic 'driver' mutations with huge intratumor differences	Mutations do not initiate cancer. 'Bad luck' mutations occur spontaneously during rapid proliferation when the CSCs and cancer cells undergo clonal expansion.
The causative role of somatic mutations is not understood.	VSELs undergo epigenetic changes and exhibit genomic instability due to exposure to extrinsic, endocrine insults. This provides a fertile ground for DNA defects (mutations) to occur when progenitors divide rapidly and undergo clonal expansion [156–159]
Genomic alterations considered hallmark drivers of specific cancers are also present in benign and premalignant conditions, at higher frequencies than in their malignant counterparts [29]	The process of asymmetrical cell divisions in VSELs and symmetrical cell divisions and clonal expansion of immediate progenitors are features of normal stem cells. Thus, mutations that are an outcome of rapid proliferation and get magnified in cancer (Fig. 3) are expected to be detected in normal tissues as well as in other pathologies.
Targeting somatic mutations or using organoids to develop personalized treatments are exciting advancements in the field [34, 35, 196]	This requires serious rethinking because both somatic mutations and organoid formation are consequences of cancer, and occur in cells undergoing rapid proliferation, and targeting them will not cure cancer since stem cells remain unaffected Cancer initiation implies epigenetic changes in immortal, pluripotent VSELs & correcting these is crucial to treat and avoid recurrence.
Cells in which mutations occur initially remain elusive. All cancer cells should have mutations but sequencing studies have reported zero mutations in several instances. There is no proven set of mutations that can transform a normal cell to a cancerous state [17, 20]	Mutations do not initiate cancer. Mutations remain random and heterogeneous since they depend on the extent of damage to the niche or the CSCs. This varies from patient to patient and this is why no fixed pattern of mutations has emerged for various solid cancers.
Many carcinogens are not mutagens. Embryonic stem cells (with no mutations) form teratocarcinoma when placed in normal tissue and become normal and functionally integrated in a developing embryo. Thus, the microenvironment can help reverse the fate of cancer cells to a normal state	VSELS are sensitive to non-mutagenic insults including the endocrine disruptors. Like embryonic stem cells, VSELs are sensitive to their microenvironment and can be manipulated and reversed back to their normal state [192, 193]
Variation in cancer risk amongst various tissues in the human body is poorly explained by the number of gene mutations [37, 72]	Cancer incidence in various tissues and geographical variation depends on the VSELs turnover and their niche. VSELs, being cell-of-origin of cancer, can also explain geographical variation in cancer incidence.
According to STDTC theory, mutations accumulate in the stem cells [37, 72]	This may not be true since VSELs normally exist in the G0 stage in a quiescent state [178, 179, 150]. Mutations occur in actively dividing CSCs/cancer cells with genome instability - during clonal expansion due to various insults - in a random manner (Fig. 3)

Precision Medicine: Should We Target Gene Mutations or CSCs To Treat Cancer?

Targeting mutations through Precision Medicine to treat cancers has proved difficult because of inter- and intra-tumor heterogeneity and a phenomenon termed 'polyclonal' or 'heterogenous' resistance that exists in tumor sites within one patient, or even amongst tumor cells within one site, complicating efforts to target resident tumors therapeutically. Moreover, metastasis is the leading cause of cancer mortality, but antitumor therapies mostly target the biology of primary tumors, with little consideration of metastases. Thus, the concept of personalized medicines remains far from global application in Clinics [196]. This is a common situation across multiple cancers. We discuss below two examples including pancreatic and lung cancer and that manipulating tissue-resident stem cells may be a better approach for cancer treatment.

Example 1: Pancreatic cancer

About 1/3 of human cancers including almost 90% of pancreatic ductal adenocarcinoma, lung and colorectal cancers are driven by mutations in the RAS gene which comprises KRAS, HRAS and NRAS. Mutated RAS gene supports uncontrolled growth of cells and evasion of apoptosis. Various approaches to block RAS gene activation have proved ineffective despite the huge interest shown by scientists and the pharma industry for more than 4 decades. The advancement of KRAS- G12C (selective inhibitor) generated lots of promise and excitement. But there is again a roadblock and patients do not respond to KRAS-G12C inhibitor therapy mainly due to intrinsic or acquired resistance and second-site mutations [197]. Hingorani [198] has published a review in Nature Reviews Cancer on pancreatic ductal adenocarcinoma and discussed the interaction of the mutated ductal epithelium with the stroma leads to tumor evolution. Attempts to abrogate myofibroblast activity by prolonged chemical or genetic approaches also do not help.

On the other hand, pluripotency markers OCT-4, SOX-2 and NANOG are reported in pancreatic cancer cell lines [199, 200] and in human pancreatic tumors [199, 201]. These markers are indeed reported in multiple cancers [11] and are negative prognostic factors connected with tumor recurrence and clinical progression [199]. These stem cells are resistant to standard oncotherapy. In the hamster model of pancreatic cancer (induced by BOP- N -nitrosobis (2-oxypropyl) amine), Iki and Pour [202] reported diffuse cytoplasmic OCT-4 expression in normal pancreas while nuclear OCT-4 was detected in large numbers of cells and small foci in the cancer samples. *These results suggest that a selective increase in VSELs resulted in pancreatic cancer since nuclear OCT-4 is a specific marker of pluripotent VSELs and is not expressed by any other cell types in the body. Cytoplasmic OCT-4 is expressed by the progenitors and under normal conditions VSELs are very few thus nuclear OCT-4 is not detected, only patchy cytoplasmic OCT-4 in progenitors was detected. An increase in Oct-4A and the disbalance between Oct-4A and Oct-4 expression reflects the cancer state.*

Various approaches are described in literature targeting the cells expressing these markers.

- Lu et al. [203] carried out a double knockdown of Oct4 and Nanog in CSCs isolated from the PANC-1 cell line and demonstrated significantly reduced proliferation, migration, invasion, chemoresistance, and tumorigenesis in vitro and in vivo. The altered expression of the genes was related to pancreatic carcinogenesis and metastasis. They suggested that Oct4 and Nanog may serve as a potential marker of prognosis and a novel target of therapy (rather than targeting mutations) for pancreatic cancer.
- Gao et al. [204] showed that miR-335 inhibits OCT4-positive tumor sphere-forming and tumor-initiating CSCs in pancreatic cancer, implying that miR-335 might play a role in the self-renewal of pancreatic cancer stem cells. Similar results were also observed in pancreatic cells, where lentiviral miR-335 restoration significantly inhibited the clonogenic growth and tumor spheres [204].
- Non-toxic natural plant products can be used to prevent and treat pancreatic cancer. Ma et al. [205] showed the potential of α-Mangostin to inhibit the expression Nanog, Oct4, c-Myc, Sox-2 and KLF4 in CSCs. Similarly, resveratrol also exerts direct effects on pancreatic stem cells [206, 207]. These results suggest that cancer can be treated/cured by improving the epigenetic state of the CSCs and it is not necessary to target mutations (that develop as a consequence). Since Nanog, Oct4, Sox-2 and KLF4 are also markers for VSELs, results suggest that α-Mangostin directly modulated CSCs/VSELs.
- MSCs have also been transplanted in patients with pancreatic cancer [208]. Molecules, vaccines, antibodies and CAR-T are being developed targeting CSC pathways [209]. However, there exist several hurdles that need to be overcome to effectively eliminate CSCs.

VSELs are reported in the mouse pancreas and their role in pancreatic regeneration after inflicting chronic injury (partial pancreatectomy) has been demonstrated [210]. Nuclear OCT-4 is a specific marker for pluripotent VSELs which

give rise to progenitors with cytoplasmic OCT-4. OCT-4 expression is eventually lost as the cells differentiate further. These stem/progenitor cells exist in few numbers in normal tissues which explains diffuse cytoplasmic OCT-4 in normal pancreas. The presence of a large number of cells with nuclear OCT-4 reported by Iki and Pour [202] is suggestive of a selective increase in nuclear OCT-4 expressing VSELs in cancer. It has been suggested that dysfunctions of VSELs possibly result in diabetes and cancer [211]. Mohammad et al. [212] showed the presence of OCT-4 positive VSELs in mouse pancreatic islets. Islets were enzymatically isolated and studied for pluripotent markers (*Oct-4, Sox-2, Nanog*) by RT-PCR. Bigger-sized, progenitor pancreatic stem cells with cytoplasmic OCT-4 and small VSELs with nuclear OCT-4 were visualized (Fig S1). Increased numbers of nuclear OCT-4 positive cells are reported in a hamster model of pancreatic cancer [213]. Nuclear OCT-4 is a specific marker for pluripotent state and probably increased numbers of VSELs were detected in the hamster model of pancreatic cancer. It is intriguing to note that the article suggested pancreatic cancer initiates from the islets [213] and we have reported VSELs in the enzymatically isolated mouse pancreatic islets [126] (Fig S1). Similar increased numbers of nuclear OCT-4 positive cells are reported in multiple human cancers [11] including testicular [214, 215] and ovarian cancer [160–162] and also in mouse models of endometrial and testicular cancer [155–158].

A fundamental conceptual change in our thinking and moving away from the concept of cancer being a genetic disease to being a stem cell dysfunction needs to be arrived at. Acknowledging the presence of VSELs, their role in maintaining normal homeostasis and getting epigenetically transformed into CSCs to initiate cancer is crucial. There is no need to target mutations, rather normalizing VSELs functions can reverse cancer if cancer gets detected early. Mutations are a consequence of cancer as shown in Fig. 3 and thus targeting gene mutations only results in the development of other, more aggressive mutations and may not ensure a cure. Once this is accepted, cancer can be 'cured' or 'reversed' when detected early, by manipulating the epigenetic state of CSCs and pushing VSELs back to the G0 state of the cell cycle. Another example to prove the hurdles associated with targeting genetic mutations is discussed next.

Example 2: Lung Cancer and Recent TRACERx Studies

The 9 years long, TRACERx (TRAcking non-small cell lung cancer evolution through therapy) studies, with a budget of 10 million pounds, resulted in 5 publications in Nature journals in 2023 and were undertaken on 421 patients with non-small cell lung cancer (NSCLCs) from 13 different hospitals in the United Kingdom. The studies were undertaken with the main objective to determine the genomic path as the cancer progresses from primary to metastatic site and the association between intratumor heterogeneity and disease-free survival. Hayes and Meyerson [36] have summarized and discussed the outcome of all these studies in their commentary. The results provided by the studies were perplexing. A lot of intra-tumor heterogeneity was observed, and cancer kept evolving into a more aggressive nature and newer mutations and clones despite chemo- or immunotherapy eventually leading to drug resistance and treatment failure. Circulating tumor DNA released by the cancer cells as well as the role of immune cells in targeting lung cancer was also studied. It was concluded that a universal cure for advanced cancer may not be possible because of extensive heterogeneity and only early detection of cancer as well as relapse is crucial for effective management. The study revealed no distinct pattern of mutations, exome or transcriptome despite all patients being affected by NSCLCs. Hayes and Meyerson [36] also questioned the role of genomic variations during metastasis and the potential for harnessing the results to target metastatic clones. Similar studies are planned to be undertaken for renal, breast and esophageal cancers soon. The study is further extended over another 7 years as TRACERx EVO with a budget of 14 million pounds to provide a deeper understanding of the evolution, prevention, and treatment of lung cancer.

Currently, actively dividing cancer cells are targeted by oncotherapy, and we suggest that this is the basic cause for the results obtained in the TRACERx studies. *Searching for mutations/genetic changes that initiate cancer, cause metastasis, and are responsible for recurrence is a mistake*. Therapeutic targeting of mutations will not cure cancer, new mutations will evolve and continuously emerge since the stem cells remain unaffected and will continue to undergo clonal expansion (Fig. 3). If a particular mutation is targeted- another one (maybe more aggressive) will evolve since rather than initiating cancer, mutations are simply a consequence of cancer (Fig. 3). Cancer is not a genetic disease but initiates due to dysfunctions of tissue-resident, pluripotent VSELs (Fig. 3). Mutations occur when the dysfunctional VSELs (with epigenetic changes) transform into CSCs and undergo rapid self-renewal and clonal expansion. CSCs are responsible for the initiation, metastasis as well and recurrence of cancer and one needs to detect and study CSCs in circulation rather than the circulating tumor cells or circulating tumor DNA released from cancer cells-based detection of seeding clones. A recent article on chimeric antigen receptor (CAR)-engineered T-cells [216] mentions absolute safety but efficacy is an issue because of existing cancer heterogeneity.

The complete lack of acknowledgment of the presence of tissue-resident stem cells (VSELs) in the lungs and their role in cancer initiation in the TRACERx studies is not justified.

VSELs are reported in the lungs [126], have a role in regular cell turnover [217], increased expression of CSCs markers (OCT-4, CD133, CD166) [218, 219], and aberrant DNA methylation is reported in lung cancers including NSCLCs [220]. The role of estradiol is reported in lung carcinogenesis and tumor progression [221]. Like the studies discussed above [155–158], early developmental exposure to endocrine disruptor bisphenol A affects lung development in macaques [222] and children [223] and resulted in lung cancer [224, 225]. It has been reported that NSCLCs initiate in non-smokers by small smoke particles sitting on cells with mutations [226] seems doubtful. It will be ideal if tissuer-esident VSELs/ CSCs can also be investigated both in the cancer tissue biopsies and in the circulation of the patients in the TRACERx EVO study to generate comprehensive data.

On the other hand, proof-of-concept studies in mice [192, 193] suggest it may be possible to reverse cancer by treating cancer subjects with epigenetic regulators and by improving the microenvironment. For this to succeed in the Clinics, early detection of cancer is crucial when it is a weak disease and the test based on stem cells in circulation has the potential for the same [11].

Key Take Home Points

Various salient points listed below were discussed above and are supported by published literature. These points are neither dogmatic nor assertive or unchallengeable but provide many future directions for research in the field including further validation of the ideas discussed here. Mid-course corrections with an open mind are crucial to addressing the growing complexity of the cancer genomic landscape. Further research is required to generate data using clinical samples.

- Cancer is initiated by endocrine/environmental insults that lead to epigenetic changes in tissue-resident VSELs. VSELs express steroid and gonadotropin hormone receptors and are thus directly vulnerable to these insults. Only VSELs, being immortal, can transmit early life insults to adult life compared to somatic cells with a limited lifespan that are regularly replaced.
- Epigenetically altered VSELs (CSCs) exhibit genomic instability and have defective DNA damage repair and DNA methylation machinery, which are fertile grounds for mutation development. Mutations detected in cancer samples are indeed bad-luck mutations, are a consequence rather than the cause of cancer, and mostly occur in proliferating progenitors (also termed adult stem cells) in agreement with earlier reports [160, 161]. Asymmetrical and symmetrical divisions and clonal expansion are characteristic features of stem/progenitor cells in normal tissues. Mutations occur because of increased self-renewal and excessive DNA replication in CSCs during rapid clonal expansion to form spheres. Altered niches and advanced age can also result in mutations during clonal expansion of stem cells in otherwise normal tissue or benign diseases such as endometriosis. Based on studies in mice, we recently reported that various uteropathies are linked and occur because of stem cell dysfunction [157].
- Because VSELs, being quiescent, do not accumulate mutations, cancer can be reversed/prevented by normalizing the epigenetic state of CSCs [192, 193]. The concepts of an epigenetic diet and the role of nutraceuticals are strongly suggestive of this.
- Logically, tissues with increased turnover and advanced age are expected to have an increased risk of developing cancer because they have age-related compromised niches and greater chances to develop epigenetic defects when VSELs undergo ACD and transition into progenitors.
- This review article is based on studies done in mice and a few clinical studies. Also, epigenetic changes studied in VSELs in normal mouse bone marrow and in response to endocrine insults are limited in nature. There is huge scope for further research including clinical studies. However, interrogating OCT-4A along with a panel of other markers in a liquid biopsy as a pan-cancer test is a huge advance at EBPL to offer early diagnosis [11, 191]. Using a causative marker to detect cancer early will be preferred over resultant markers for cancer detection, metastasis, and recurrence.
- Rather than genetic control of cellular functions, epigenetic control of stem cell behavior needs to be appreciated and targeted to win the war against cancer and achieve the Cancer Moonshot.
- Cancer is a stem cell disease and reversible if detected early [10, 192, 193].

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Declarations

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