Review

Malignances Stage Development in vitro: The Role of (Very) Small Cancer and Leukemic Stem-Like Cells

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Review

Malignances Stage Development *In Vitro*: The Role of (Very) Small Cancer and Leukemic Stem-Like Cells

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Simple Summary: This review explores the origin of Leukemic Stem-Like Cells (LSLCs) from (Very) Small Leukemic Stem-Like Cells ((V)SLSLCs) and Cancer Stem Cells (CSCs) from transformed Very Small Embryonic-Like Stem Cells (VSELSCs). Understanding this relation is a step toward uncovering the mechanisms behind cancer and leukemia initiation and development. We explore how these very small precursor cell stages might contribute to the initiation and progression of cancer as well as leukemia with a focus on Acute Myeloid Leukemia (AML) as a model.

Abstract: Recent experimental findings indicate that CSCs originate from transformed VSELSCs, which are early precursor stages of healthy stem cells. This discovery marks a crucial step in understanding the mechanisms that drive cancer development and initiation. Our team's follow-up studies on leukemia, lung cancer, and healthy embryonic kidney cells have, for the first time, identified stages resembling very small precursor stem cells in continuously growing cell lines. These findings suggest the existence of (Very) Small Cancer Stem Cells ((V)SCSCs) or (V)SLSCs within respective subpopulations of CSCs and LSCs *in vivo*. In this review, we explore theoretical examples of how leukemia progresses through various stages, highlighting the role of these very small precursor cells as fundament primitive stages. We also discuss the potential implications of further research into these unique cellular stages for advancing cancer and leukemia treatment and prevention strategies.

Keywords: very small embryonic-like stem cells; leukemic stem cells; (very) small cancer stem-like cells; (very) small leukemic stem-like cells; precursor-like stages; *In vitro* studies; cancer development; leukemia development; cellular transformations

1. Introduction

The CSC hypothesis was first proposed in the 1990s [1]. Since then, it has been the subject of extensive research [2], contributing to a better understanding of tumorigenesis and the development of more effective cancer therapies [3].

Stem Cells (SCs) are undifferentiated, primitive cells present within an organism [4]. When cultured in vitro, these cells often undergo epigenetic changes and exhibit dysfunctions, thus they are better described as Stem-like Cells (SLCs) [5–10]. In cancer research, a subset of these cells, known as CSCs, has been identified. Outside the organism, these cells are termed Cancer Stem-like Cells (CSLCs) [2]. CSCs and CSLCs are typically malignant cells originating from epithelial tissues. Malignant cells from other germ layers have specific names; for example, in leukemia, they are called Leukemic Stem Cells (LSCs) [1].

Initially, the CSC hypothesis was considered controversial [11–13], but over time, and is now widely accepted [14–16]. Among CSCs, their precursor stages have garnered significant interest and

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hope exploration of the mechanisms of carcinogenesis [17]. Bhartiya et al. in 2023 described experimental results indicating that CSCs originate from transformed VSELSCs [17]. The existence of CSCs from transformed VSELSCs, exemplified by (Very) Small Cancer Stem-Like Cells ((V)SCSLCs) and (V)SLSLCs, was confirmed a few months later that same year [18]. It's noteworthy that VSELSCs were discovered in 2006 [19], and like CSCs and SCs, they were initially met with skepticism [20]. To date, many independent laboratories have confirmed the presence of VSELSCs *in vivo* [21], and recent reports about their cell culture counterparts have generated considerable interest [18]. This uniquely addresses communication about their *in vitro* analogs given the availability of experimental material in the form of cell cultures and their enrichment by efficient methods in the number of primitive stages [18,22–24]. There is a real possibility for comparing the healthy and malignancy most primitive stages by obtaining a minimum of 5000 (10000 preferred) cell stages for single-cell RNA sequencing analysis. These results enhance our chances of understanding the molecular mechanisms underlying malignant proliferation growth, as well as differences in cell cycle checkpoints [25] and cell stage division types according to the Hayflick phenomenon [26] between healthy VSELSCs and transformed (V)SCSCs as well as (V)SLSCs, and their stage analogs *in vitro*.

Table 1. Very Primitive Cellular Stages *in vitro* and *in vivo*. * Suggested names.

Cellular Stages	
In Vivo	***In Vitro***
Stem Cells, SCs	Stem-Like Cells, SLCs
Cancers Stem Cells, CSCs	Cancers Stem-Like Cells, CSLCs
Leukemic Stem Cells, LSCs	Leukemic Stem-Like Cells, LSLCs
Very Small Embryonic-Like Stem Cells, VSELSCs, VSEL	(Very) Small Embryonic-Like Stem Cells, (V)SELSCs
*(Very) Small Cancer Stem Cells, (V)SCSCs	(Very) Small Cancer Stem-Like Cells, (V)SCSLCs
* Small Cancer Stem Cells, SCSCs	Small Cancer Stem-Like Cells, SCSLCs
* Small Leukemic Stem Cells, SLSCs	Small Leukemic Stem-Like Cells, SLSLCs

2. Current Knowledge

The skepticism surrounding the existence and role of VSELSCs is similar to the initial doubts about SCs and CSCs [1,2,11–13]. However, identifying VSELSCs is more complex due to their size and morphological similarities to extracellular vesicles (EVs) and apoptotic bodies (ABs). These similarities make it challenging to distinguish VSELSCs from cellular debris in scatter dot plots [27]. Despite these challenges, EVs [28–35], ABs, and VSELSCs are all significant in various biological processes. However, the ability to self-renew, grow, and proliferate is unique to VSELSCs [36–38]. Given the morphological similarities among these structures, we provide an overview of their distinct characteristics and summarized on Table 2.

Table 2. Cytological characteristics and comparison of the very primitive cell stages with the cellular debris. Aberrative: VSELSCs - Very Small Embryonic-Like Stem Cells, (V)SLSLCs - (Very) Small Leukemic Stem-Like Cells, LSLCs - Leukemic Stem-Like Cells, ABs - Apoptotic Bodies, EVs - Extracellular Vesicles. Information about (V)SLSLCs was sourced from [21], while data on LSLCs was obtained from [18,22,23].

	Type of Event				
Cytological Parameter	**VSELSCs**	**(V)SLSLCs**	**LSLCs**	**ABs**	**EVs**
Median size (diameter)	around 5-7 μm	around 5-7 μm	around 9-12 μm	1-5 μm	0.1-1 μm
Membrane integrity	preserved	preserved	preserved	unusual	unusual
Specific cell surface markers	yes	yes	yes	unusual	unusual
Cytoplasm	yes	yes	yes	unusual	yes
DNA	yes	yes	yes	yes	unusual
Cell fusion/absorption	yes	yes	yes	yes	yes
Self-renewal	yes	yes	yes	no	no

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2.1. Cytological Characteristics of Extracellular Vesicles

EVs are tiny, membrane-enclosed particles released by cells during various biological activities [39–44]. Their size and function vary depending on their cell of origin, which affects their environmental interactions and role in cell-to-cell communication [39–44]. EVs transport a variety of molecules, including proteins, lipids, nucleic acids, and signaling compounds, through interactions facilitated by their membranes. However, they are not always completely isolated from the external environment by their lipid membrane [39–44]. Different types of EVs include:

Ectovesicles- These protrude from the cell surface and may assist in intercellular signaling;

Exosomes- Ranging from approximately 30 to 150 nanometers in size, exosomes can be recognized by specific membrane markers and proteins;

Microvesicles- Typically measuring between 0.1 and 1 micrometer, these are released by cells in response to activation, oxidative stress, or inflammation;

Apoptosomes- Varying in size and composition based on the apoptosis stage and cell type, they are surrounded by a lipid membrane containing cellular fragments like nuclear debris or organelles [39–44]. The structural similarity between these EV subtypes and apoptotic bodies, which also include certain cytoplasmic elements, implies that they could be classified as a form ABs, and vice versa, ABs might be classified as EVs.

2.2. Cytological Characteristics of Apoptotic Bodies

ABs are typically small, circular or oval structures, ranging from 1 to 5 micrometers in diameter, and are formed during apoptosis, the programmed cell death process [45–51]. During apoptosis, the cell's chromatin undergoes significant condensation [45–51]. As the cell advances through apoptosis, it shrinks and its fragmented components are encapsulated within vesicles, which may be enveloped by a double membrane. This membrane offers some level of protection to the contents but does not ensure complete integrity [45–52]. Early-stage apoptotic bodies may not show positive staining for 7-AAD, reflecting complete isolation [53]. Importantly, this study does not cover VSELSCs and their morphology. Furthermore, the outer membrane of ABs can display specific CD surface markers, although this is not consistent across all apoptotic bodies [45–51]. The presence of these markers can vary depending on the cell type, stage of apoptosis, and other conditions, making it a characteristic with limited specificity.

2.3. Cytological Characteristics of Very Small Embryonic-Like Stem Cells

Human VSELSCs are small cellular structures, typically 5-7 micrometers in size, with unique embryonic characteristics such as the ability to self-renew and differentiate into various cell types [54–56]. Advocates for VSELSCs believe these cells could play a significant role in regeneration and have therapeutic potential [54–56]. They have been found in the peripheral blood of individuals across different age groups, from young to elderly [57]. However, critics argue that the lack of clear definitions and universally accepted markers makes it difficult to distinguish VSELSCs from other cell types or cellular debris. While the concept of VSELSCs is promising, more detailed research is needed to understand their biological mechanisms and the accuracy of detection methods. Despite these challenges, there is a growing body of independent studies confirming the existence of VSELSCs. Cellular debris, which consists of cell fragments from disintegration, apoptosis, or other cellular activities, can be mistaken for living cells during flow cytometry analysis [58].

2.4. Cytological Characteristics of Very Small Leukemic Stem-Like Cells

Identifying specific CD markers that uniquely target SCs, CSCs, LSCs, and their precursors is challenging [56]. These cells might fuse with other cells, avoiding detection and potentially entering the external environment after cell death [59–64]. Because of their morphology, they can be mistaken for EVs and ABs in some assays, and vice versa. The involvement of EVs and ABs in cellular development is an area of intense research, as many studies indicate [65–68]. Very small cellular debris and EVs generally appear in scatter dot plot regions that are hard to count. Rarely, EVs and

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ABs can mimic (V)SLSLCs, being a few micrometers in size and containing DNA fragments similar to cell nuclei. Differentiating them from (V)SLSLCs is aided by a round membrane, pH environment, and mature chromatin. The use of 7-AAD dye in samples helps distinguish them on scatter dot plots from events with intact membranes. Since some EVs and ABs are similar in size and content, can display CD markers, and in early stages may not allow the entry of 7-AAD and similar dyes, distinguishing them from (very) small cell developmental stages remain challenging.

3. Proposed Cascade of Stage Transformation of Acute Myeloid Leukemia

Here, we propose models that outline potential pathways of development for (V)SELSCs (Figure 1), shedding light on their role in acute myeloid leukemia (AML) initiation (Figures 2-3). One scenario posits that only (V)SELSCs possess self-renewal capabilities, triggering asymmetric divisions upon reaching a critical cell density, resulting in the generation of SELSCs. Subsequent asymmetric divisions of SELSCs give rise to SLCs. Alternatively, another model suggests that (V)SELSCs, SELSCs, and SLCs all exhibit self-renewal abilities, leading to the formation of self-renewing SESLCs, which then differentiate into SLCs. SLCs undergo self-renewal and, upon reaching a critical cell density, initiate asymmetric divisions to produce multi- or oligopotent progenitors. In this model, each of the highly primitive precursor stages has the capability to form colonies. Their release triggers self-renewal and asymmetric division in the parental colony. These models underscore the complexity of (V)SELSCs developmental transformations and highlight the need for further research to elucidate their precise contributions to cancer or leukemia initiation and progression (Figure 1). Similarly, examples of AML origin from (V)SELSCs outline various scenarios of leukemia initiation, emphasizing the interplay between (V)SELSCs, (V)SLSLCs, SELSCs, SLCs, and LSLCs at different stages (Figure 2). The inclusion of fusion events in these models further underscores the intricate nature of AML pathogenesis and warrants exploration in future studies to comprehensively understand the mechanisms driving leukemia development (Figure 3).

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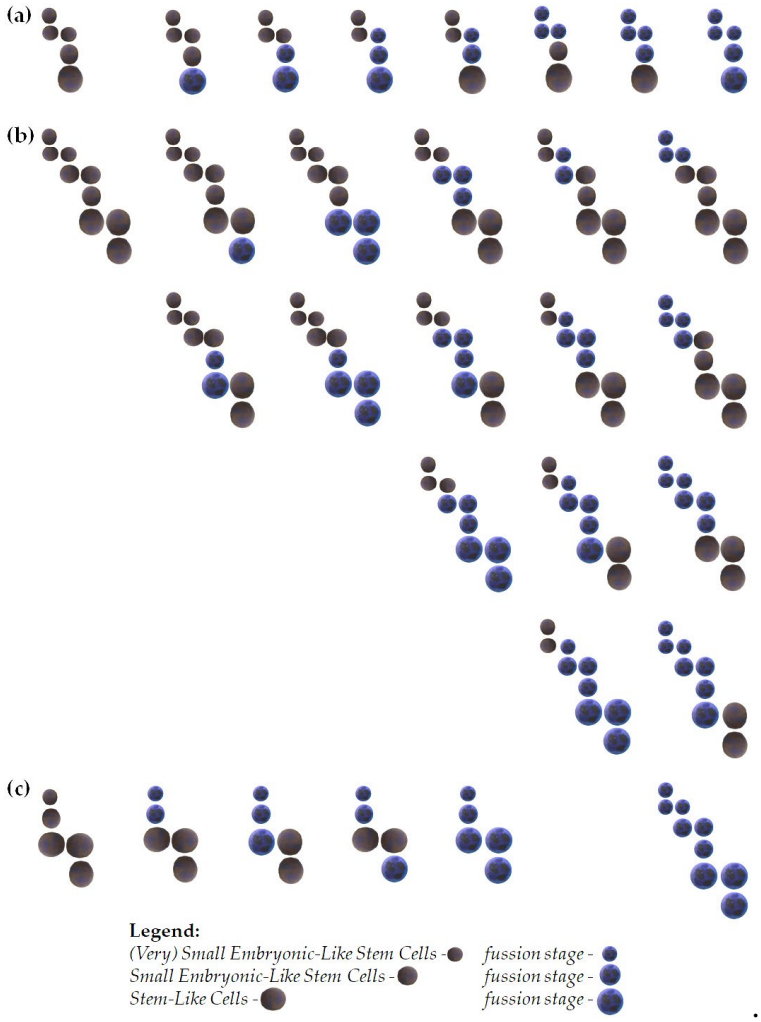

Figure 1. Proposed examples of the stage development of (V)SELSCs. (**a**) In this model, only (V)SELSCs can self-renew. Once they reach a critical cell density, they start asymmetric divisions, which produce SELSCs. The asymmetric divisions of SELSCs lead to the formation of SLC); (**b**) In this model, (V)SELSCs, SELSCs, and SLCs all can self-renew. After reaching a critical density through self-renewal, (V)SELSCs initiate asymmetric division to form SESLCs. Self-renewing SESLCs, upon reaching critical cell density, start asymmetric divisions to produce SLCs. Similarly, SLCs also self-renew and, upon reaching a critical cell density, initiate asymmetric divisions to produce multi- or oligopotent progenitors; (**c**) This model assumes that (V)SELSCs and SELSCs divide asymmetrically, and replication of these cells might occur during cells fusion; In the proposed cell fusion models, we assumed that all cells within a given stage, as well as the first and last cell stages, are capable of fusion - middle stage was not used in this model, however its self-renewal is also probable; It is possible that SESLCs might be a form of (V)SELSCs undergoing self-renewal or asymmetric divisions, and there may be other intermediate stages not included in the presented examples; It is also not excluded that very small or small precursor stages represent a certain form of "dormancy," and their activation into life processes occurs after fusion with a functioning cell.

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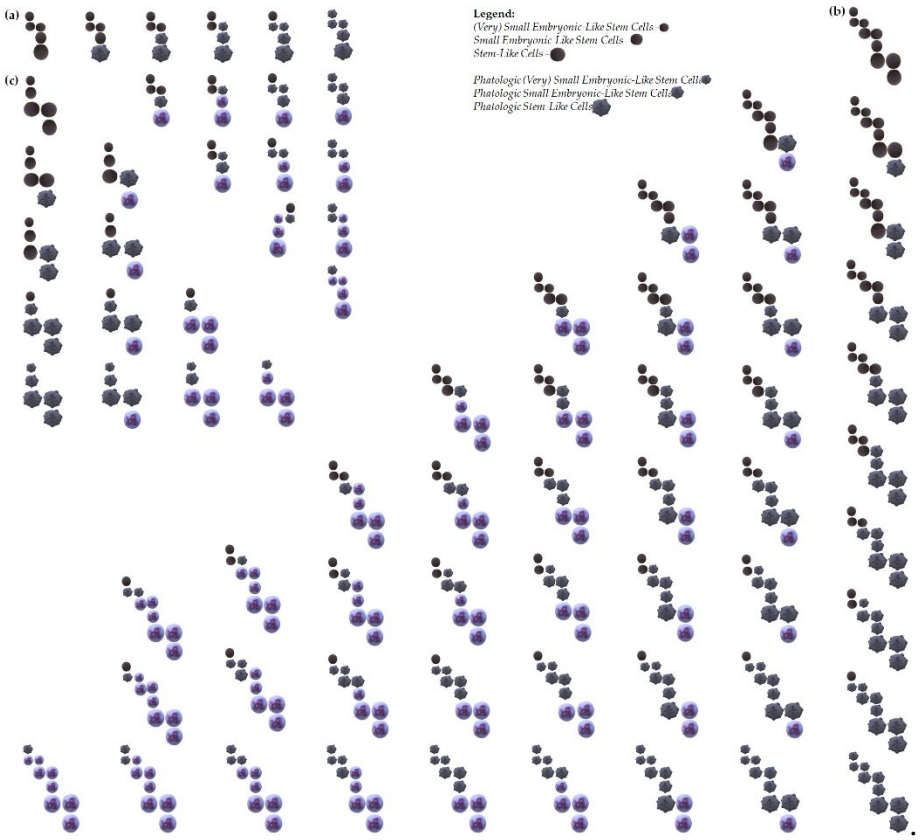

Figure 2. Proposed examples of acute myeloid leukemia origin from (V)SELSCs. (**a**) Leukemia initiation through the accumulation of changes in pathogenic/pathological cells in a model where the self-renewing stage are (V)SELSCs; (**b**) Leukemia initiation through the accumulation of changes in pathogenic/pathological cells in a model where the self-renewing stages are (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs as well as SLCs and LSLCs; (**c**) Examples of leukemia initiation in a model where the self-renewing stages are (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs, as well as LSLCs; Leukemia initiation through the accumulation of changes in pathogenic/pathological cells in a model where the self-renewing stage are (V)SELSCs; This model assumes that (V)SELSCs and SELSCs divide asymmetrically, and replication of these cells might occur during LSLCs self-renewing; It is possible that SELSCs and/or LSLCs might be a form of (V)SELSCs and/or (V)SLSLCs undergoing self-renewal or asymmetric divisions, and there may be other intermediate stages not included in the presented examples; It is also not excluded that very small or small precursor stages represent a certain form of "dormancy," and their activation into life processes occurs after fusion with a functioning cell.

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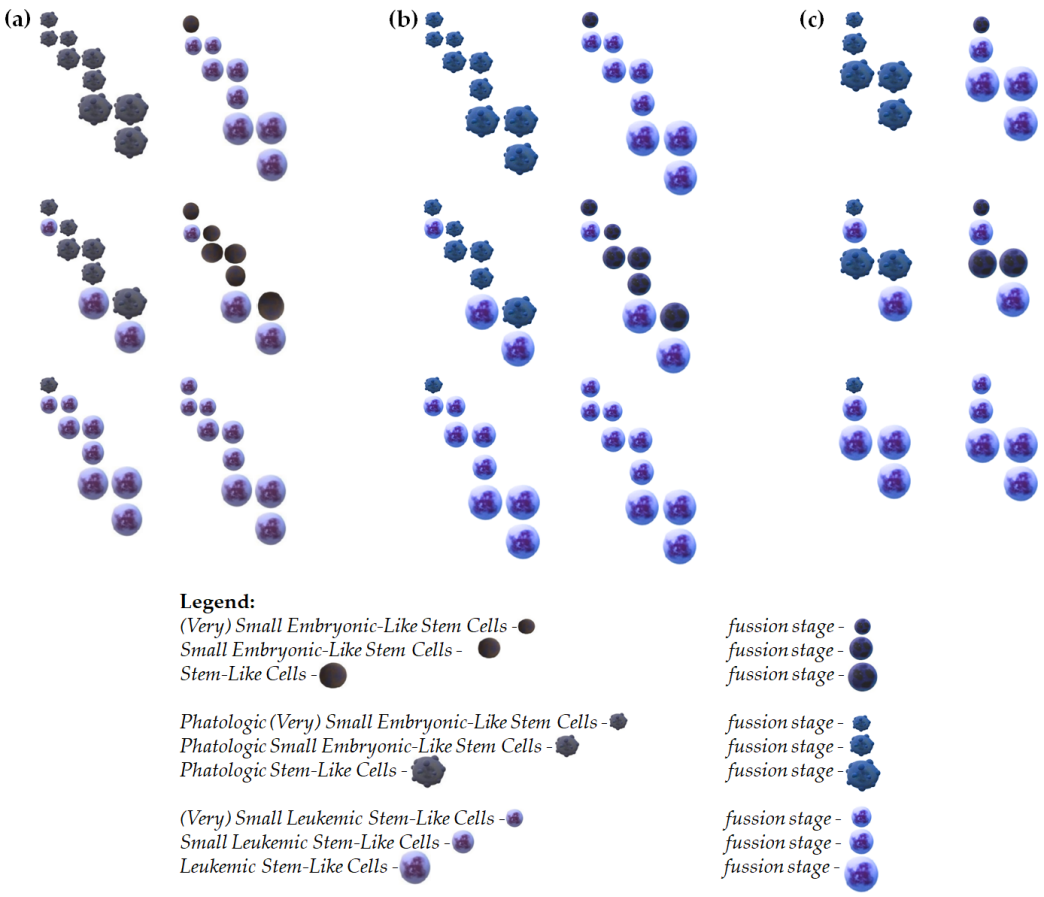

Figure 3. Proposed examples of acute myeloid leukemia origin from very primitive fusions cell stages. (**a**) - *left panel* - Leukemia initiation through the accumulation of changes in pathogenic/pathological cells at different stages in a model where the self-renewing cells are: (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs as well as SLCs and LSLCs. - *right panel* - Leukemia initiation at different stages in a model where the self-renewing stages are: (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs as well as SLCs and LSLCs; (**b**) - *left panel* - Leukemia initiation through the accumulation of changes in pathogenic/pathological cells at different stages in a model where the self-renewing and fusion stages are: (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs as well as SLCs and LSLCs. - *right panel* – Leukemia initiation at different stages in a model where the self-renewing and fusion stages are: (V)SELSCs and (V)SLSLCs, SELSCs and SLSLCs as well as SLCs and LSLCs; (**c**) - *left panel* - Leukemia initiation through the accumulation of changes in pathogenic/pathological cells at different stages in a model where that (V)SELSCs and SELSCs divide asymmetrically, and replication of these cells might occur during LSLCs self-renewing; - *right panel* - Leukemia initiation at different stages in a model where that (V)SELSCs and SELSCs divide asymmetrically, and replication of these cells might occur during LSLCs self-renewing; In the proposed cell fusion models, we assumed that all cells within a given stage, as well as the first and last cell stages, are capable of fusion - middle stage was not used in this model, however its self-renewal is also probable; It is possible that SELSCs and/or SLSLCs might be a form of (V)SELSCs and/or (V)SLSLCs undergoing self-renewal or asymmetric divisions, and there may be other intermediate stages not included in the presented examples; It is also not excluded that very small or small precursor stages represent a certain form of "dormancy," and their activation into life processes occurs after fusion with a functioning cell.

4. Benefits from Future Research Findings

4.1. Preclinical and Clinical Study

In preclinical research, considering (V)SLSLCs can assist in identifying the most effective potential chemotherapeutic agent by testing IC50 values for cell viability [22]. These findings are

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relevant in both preclinical and clinical trials (if their in vivo counterparts are included), aiding in the selection of the most efficient and least toxic drug. Moreover, such studies may enhance our understanding of metabolic pathways applicable in therapy.

4.2. Potential Clinical Applications

Targeted therapies: Identifying specific markers and mechanisms that lead to the transformation of VSELSCs and (V)SELSCs into cancer cells could enable the development of targeted therapies. Such therapies could precisely attack cancer cells at early stages of their development, potentially being more effective than current treatments.

Early diagnosis: Charatreristics of VSELSCs and (V)SELSCs as precursors to CSCs and LSCs could lead to the development of new biomarkers for earlier cancer detection. Early diagnosis is crucial for effective treatment and improved patient outcomes. New therapeutic targets: Identifying and characterizing VSELSCs as precursors to CSCs and LSCs opens new therapeutic opportunities. This research could lead to the development of therapies that are more effective in eliminating cancers at their earliest stages.

Tissue regeneration: Since VSELSCs have the ability to differentiate into various cell types, they might be used in regenerative medicine to repair of damaged tissues. This application could be relevant not only for cancer treatment but also for other conditions requiring tissue regeneration.

Resistance to treatment: Research on VSELSCs and (V)SELSCs could help elucidate the mechanisms by which cancer cells resist treatment. Understanding how these cells evade therapies could lead to new strategies to overcome this resistance.

4.2. Molecular and Epigenetic Mechanisms in the Transformation of VSELSCs to CSCs

The transformation of Very Small Embryonic-Like Stem Cells (VSELSCs) into Cancer Stem Cells (CSCs) involves a multifaceted process driven by genetic and epigenetic alterations. VSELSCs, which express hormone receptors and are susceptible to environmental insults, can undergo global hypomethylation and loss of imprinting at specific loci, leading to their conversion into CSCs [69–72]. Epigenetic changes, such as dysregulated DNA methyltransferases and altered expression of stemness markers like Oct-4A, Sox-2, and Nanog, play a crucial role in this transformation process [69–72]. The transition from quiescent VSELs to proliferative CSCs involves acquiring a cancer stem cell phenotype, genomic instability, and the activation of oncogenic pathways, ultimately contributing to cancer initiation and progression [69–72]. Targeting these molecular mechanisms offers a promising approach to prevent or reverse the development of CSCs and combat cancer effectively [69–72].

4.3. Extend the Konwledge Abaout Cancerogenesis - Epigenetic Analysis of Cells

Filling knowledge gaps: Previous research on CSCs has primarily focused on more developed cancer cell stages. Studying VSELSCs, which are earlier precursors (quite possibly the most primitive cell stage in young, middle-aged and aged human organisms [73]), can provide new insights into the initial stages of carcinogenesis, which are currently poorly understood. Comparison of healthy and cancerous cells: Research on the differences between healthy VSELSCs and their cancerous counterparts (V)SCSLCs and (V)SLSLCs can reveal key molecular and epigenetic mechanisms responsible for cancer transformation. This can aid in identifying new diagnostic markers and therapeutic targets.

Gene regulation mechanisms: Epigenetics studies changes in gene expression that are not caused by changes in the DNA sequence. Epigenetic analysis of VSELSCs can reveal which epigenetic changes lead to their transformation into cancer cells. Marker identification: Epigenetic markers can be used to identify early stages of cancer development. For example, DNA methylation and histone modifications specific to VSELSCs could serve as biomarkers for early diagnosis. Therapy development: Understanding the epigenetic mechanisms regulating VSELSCs function could lead to the development of new epigenetic therapies that can reverse or inhibit cancer transformation.

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4.4. Interactions Between Most Primitive Cancer and Healthly Precursors with the Cellular Microenvironment

Impact of the microenvironment: The cellular microenvironment plays a key role in regulating the growth, survival, and differentiation of cancer cells. Understanding how VSELSCs interact with surrounding cells can reveal mechanisms that promote or inhibit carcinogenesis.

Adaptation capability: Studying interactions between these cells and their microenvironment can reveal how cells adapt to different conditions. This is important for understanding how cancers develop resistance to treatment and how this phenomenon can be overcome.

New therapeutic strategies: Interactions between cells and the microenvironment can be targeted by new therapies. For example, disrupting signals between VSELSCs or (V)SELSCs and surrounding cells could be an effective strategy in cancer treatment.

5. Conclusions

Drawing an analogy to developmental stage-dependent transformations, consider the study of silkworm (Bombyx mori) instars under controlled conditions that shorten their developmental cycle. Even with this accelerated development, silkworms still progress through the stages of egg, caterpillar, pupa, and moth. However, the duration of each stage is reduced; for example, caterpillars can transition into pupae without undergoing the complete range of instars [74–78].

If VSELSCs serve as precursors to SCs and CSCs, it is highly probable that analogous precursor cell stages would be present in continuously growing cell cultures, despite the accumulation of various changes and alterations.

In cancer cell cultures in vitro, healthy stages of (very) small precursor stages are rather absent. However, homologous stages of VSELSCs are observed instead. This suggests that this developmental stage is highly likely to be cancerous. If this is the case, it is equally probable that these stages may also be diseased in vivo.

Given the availability of experimental material in the form of cell cultures, their increased proliferation rate compared to healthy cells, and the efficient method of enriching the cultures with primitive stages, there is a real possibility of obtaining enough cells for single-cell RNA sequencing (transcriptomics). This analysis requires a minimum of 5000 to 10000 cells to initiate the reaction. These results enhance our chances of understanding the molecular mechanisms underlying malignant proliferation growth, as well as differences in cell divisions (the Hayflick phenomenon) between healthy VSELSCs and (V)SELSCs and transformed (V)SCSCs and (V)SCSLCs as well as (V)SLSCs and (V)SLSLCs.

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