HHS Public Access

Author manuscript

Pharmacol Ther. Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

Pharmacol Ther. 2019 December ; 204: 107398. doi:10.1016/j.pharmthera.2019.107398.

Environmental exposures, stem cells, and cancer

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Abstract

An estimated 70–90% of all cancers are linked to exposure to environmental risk factors. In parallel, the number of stem cells in a tissue has been shown to be a strong predictor of risk of developing cancer in that tissue. Tumors themselves are characterized by an acquisition of “stem cell” characteristics, and a growing body of evidence points to tumors themselves being sustained and propagated by a stem cell-like population. Here, we review our understanding of the interplay between environmental exposures, stem cell biology, and cancer. We provide an overview of the role of stem cells in development, tissue homeostasis, and wound repair. We discuss the pathways and mechanisms governing stem cell plasticity and regulation of the stem cell state, and describe experimental methods for assessment of stem cells. We then review the current understanding of how environmental exposures impact stem cell function relevant to carcinogenesis and cancer prevention, with a focus on environmental and occupational exposures to chemical, physical, and biological hazards. We also highlight key areas for future research in this area, including defining whether the biological basis for cancer disparities is related to effects of complex exposure mixtures on stem cell biology.

Keywords

carcinogenesis; development; epigenetics; reprogramming; chemical; prevention

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[5.]Conflicts of Interest

The authors have no conflict of interest to declare.

1. Introduction

Early observations by pathologists identified gross similarities between embryonic tissues and tumors, leading to the original hypothesis that cancers arise from embryo-like cells (Récamier, 1829; Virchow, 1855). Further work, particularly the seminal research of Becker, McCulloch, and Till in the 1960s, provided the first evidence for tissue-specific stem cells and led to the hypothesis that tumors may arise from dysregulation of this cell population (Becker, McCulloch, & Till, 1963; Till & McCulloch, 1961). Experimental evidence accumulating over the past three decades has shown that within many tumors, the cellular hierarchy imitates that found during the development of normal tissue. Advances in single cell profiling techniques, such as fluorescence activated cell sorting, single cell transcriptomic profiling (scRNA-seq), and single cell epigenetic analyses have confirmed pathological observations of tumor heterogeneity, identifying that cancers are comprised of distinct cellular compartments that can be differentiated based on cell surface markers or activated biological pathways. These same techniques have allowed for these different tumor cell populations to be purified, assayed, and characterized. Cancer stem cells (CSCs) at the apex of the tumor hierarchy can differentiate to provide the complex cellular hierarchy found in a tumor (Wicha, Liu, & Dontu, 2006). CSCs are also defined by their ability to initiate a new tumor, suggesting an essential role in metastasis. These cells have become an intense subject of research (Kreso & Dick, 2014). CSCs have natural defense mechanisms, including increased adaptation to oxidative stress and drug resistance, which makes them resistant to traditional chemotherapeutics (Conley et al., 2012; Dean, Fojo, & Bates, 2005). In solid tumors, CSCs, similar to normal stem cells, are rare cells that are often quiescent. As chemotherapeutics often target rapidly cycling cell populations, the stem cell population of the tumor can survive, despite reductions in tumor bulk. The identification of these rare cells that potentially drive tumorigenesis highlights the need to better understand the role of environmental factors in altering cellular differentiation and promoting stemness in the context of carcinogenesis.

STEM CELLS, PROGENITOR CELLS, AND DIFFERENTIATION

Stem cells during development—Stem cells and their unique properties play a fundamental role in development, transforming a single fertilized egg into hundreds of cell types comprising the complex tissues and organs of the human body. The key properties which characterize a stem cell are *self- renewal*, the ability to reproduce cells with the same potency as themselves, and *differentiation potential*, the ability to divide and give rise to differentiated offspring (Gupta, Pastushenko, Skibinski, Blanpain, & Kuperwasser, 2019). Potency is a stem cell's potential to differentiate into various cell lineages. Over the course of embryonic development cells lose potency the further down the hierarchy they differentiate (Figure 1).

The top of the hierarchy is occupied by *totipotent stem cells*, capable of differentiating into all cell types, both embryonic and extraembryonic. Totipotent stem cells give rise to *pluripotent stem cells* with the ability to differentiate into all cell types arising from the three germ layers, the endoderm, ectoderm, and mesoderm (Singh, Saini, Kalsan, Kumar, & Chandra, 2016). Further progressing down the hierarchy*, multipotent, oligopotent,*

unipotent, and stem cell progenitors all maintain self-renewal capabilities, but have significantly limited differentiation potential compared to their predecessors. Multipotent stem cells can differentiate into cell types within a close cell family, and oligopotent, unipotent, and progenitors are able to produce progeny within the same lineage (Hayes, Curley, Ansari, & Laffey, 2012). Traditionally, the unilateral differentiation down the hierarchy was viewed as the dogma of embryonic development, however research in the last decade challenges this, proposing more fluid and multi-directional differentiation behavior along the hierarchy.

Stem cells in adult tissue homeostasis and wound repair—Long after embryonic development, stem cells play a crucial role in the body, primarily through *adult tissue homeostasis*, the maintenance of a constant number of healthy cells in an organ, and *wound repair,* the dynamic process of restoring damaged tissue (Goichberg, 2016; Shaw & Martin, 2016). Adult tissue homeostasis is necessary to ensure optimal organ function, and tissue stem cells differentiate into the specific cell types needed to replace the cells lost and damaged from general “wear and tear”. This is especially important in tissues such as the epidermis and its appendages and the intestinal epithelia, both of which are highly vulnerable to environmental stressors, resulting in rapid turnover (Ojeh, Pastar, Tomic-Canic, & Stojadinovic, 2015; Visvader & Clevers, 2016). In a number of tissues, including the well characterized epidermis and small intestine, stem cells reside in *stem cell niches*, localized microenvironments where stem cell populations are maintained and stored until activation (Morrison & Spradling, 2008). The location of niches exemplifies the importance of stem cells in homeostasis as they are functionally housed in deep, well protected areas within a tissue. Examples of this include the aptly named crypt base columnar cells (CBCs) located at the bottom of the intestinal crypt, their highly debated neighbors at the “+4” position above the base, stem cells located in the basal layer of the epidermis, and hair follicle stem cells (hfSCs) located in the “bulge” at the base of the hair follicle (Nick Barker, 2014; Morrison & Spradling, 2008; Ojeh et al., 2015; Sakthianandeswaren et al., 2011). During homeostasis, these stem cells migrate and differentiate up and outwards to replenish lost and damaged cells. Instead of passively waiting in the niche until they are needed for replacement, stem cells are highly active in sensing and signaling to their surroundings and neighboring cells as well as maintaining their own population through self-renewal. Key developmental pathways Wnt and Notch have been implicated in these homeostatic processes (Clevers, Loh, & Nusse, 2014; Morrison & Spradling, 2008; Sancho, Cremona, & Behrens, 2015). These events display the dynamic process of homeostasis and the high levels of signaling and regulation, involved in ensuring proliferation and differentiation of stem cells at the appropriate time, number, and location (Biteau, Hochmuth, & Jasper, 2011).

In addition to homeostasis, adult tissue stem cells are crucial in regenerating tissues after injury. Stem cells are crucial to wound repair due to their capability to migrate to the site of tissue injury, rapidly proliferate, and to differentiate into the necessary cell types to restore the damaged tissue (Goichberg, 2016; Shaw & Martin, 2016). Following injury, stem cells in the hair follicle bulge, which do not migrate to the epidermis under normal conditions, migrate to epidermal sites of injury and aid in re-epithelializing wounds (Chou et al., 2013; Ito et al., 2005). Additionally, after migration and re-epithelialization, hfSCs expressed an

epidermal phenotype but were eliminated in the tissue after several weeks, displaying their targeted migration to aid in wound repair. In contrast, muscle stem cells which are primarily quiescent compared to hfSCs, have also been shown to rapidly migrate and differentiate into regenerative myofibers following tissue injury, highlighting the importance of stem cell involvement in wound repair across multiple tissue types (Dumont, Bentzinger, Sincennes, & Rudnicki, 2015).

Wound repair is a highly dynamic process involving the coordination of chemical signaling, cell migration, and regulated growth and differentiation. The wound healing process is often broken up into three major phases—inflammation, proliferation, and remodeling. Mesenchymal stem cells (MSCs) are involved in all three stages, migrating to damaged tissue and interacting with the stroma and macrophage signaling. (Maxson, Lopez, Yoo, Danilkovitch-Miagkova, & LeRoux, 2012). As the first phase of the wound repair process, inflammation is an especially critical step as it guides the signaling and recruitment of effector cells necessary to execute downstream reconstruction of the extracellular matrix, angiogenesis, and remodeling to normal tissue. Inflammation is a self-limiting process, reliant on a highly sensitive balance of pro-inflammatory and anti-inflammatory cytokines, chemokines, and growth factors, where disruption of this balance has the potential to result in pathologies such as chronic inflammation and neoplasia (Coussens & Werb, 2002). MSCs and macrophages are especially important in helping wounds progress past the inflammation phase as MSCs decrease pro-inflammatory cytokines (TNF-α and IFN-γ) and increase production of anti-inflammatory cytokines (IL-10 and IL-4) and macrophages clear damaged and apoptotic leukocytes, resulting in the resolution of inflammation and allowing the wound to progress to the proliferation phase(Guo & DiPietro, 2010; Maxson et al., 2012). During proliferation and remodeling, MSCs release growth factors FGF, VEGF, PDGF, TGF-ß, and others to recruit fibroblasts, keratinocytes, and host stem cells to reconstruct the extracellular matrix and re-epithelialize the wound (Shi et al., 2012).

Chronic inflammation is a characteristic of the tumor microenvironment and is becoming increasingly implicated as a major mechanism involved in the initiation and progression of tumorigenesis. In the tumor microenvironment, inflammatory molecules such as IL-6, IL-8, TGF-β1, NFκβ, TNFα, and HIF-1α regulate proliferation and metastasis (Cabarcas, Mathews, & Farrar, 2011; Plaks, Kong, & Werb, 2015). The tumor microenvironment also works to actively immunosuppress natural killer (NK) cells and CD8+ T cells in order to allow continuous inflammation and tumor progression, which would otherwise be stopped by these cytotoxic cells (Plaks et al., 2015). The inflammatory infiltrate in tumors is maintained by a precise balance of pro-inflammatory and anti-inflammatory cytokines that stimulate angiogenesis and neoplastic growth (Coussens & Werb, 2002). Where normal tissues exhibit a highly organized structure utilizing angiogenesis and differentiation in wound repair to restore tissue organization, angiogenesis and neoplastic growth in invasive carcinomas occur in a highly disorganized manner reflecting a chaotic remodeling of the extracellular matrix. Indeed, the tissue reorganization field theory of cancer posits that alterations of the stroma and disrupted stromal/epithelial interactions are likely major drivers of carcinogenesis (Soto & Sonnenschein, 2011). More specifically, Soto and Sonnenschein show in multiple *in vitro* studies utilizing various cell lines that the dynamic interaction between mammary epithelial cells and the breast stroma is crucial to proper mammary gland

morphogenesis and that this development is highly sensitive to alterations in structure and composition of the breast extracellular matrix (Barnes et al., 2014; Dhimolea, Soto, & Sonnenschein, 2012; Krause, Maffini, Soto, & Sonnenschein, 2010). Additionally, chronic inflammation results in the accumulation of damaging radicals such as reactive nitrogen and oxygen species secreted by macrophages and neutrophils during phagocytosis(Dedon & Tannenbaum, 2004; Kvietys & Granger, 2012). In a state of chronic tissue damage and regeneration, these reactive species have prolonged opportunity to interact with DNA and cause permanent damage and mutations, the accumulation of which could potentially lead to tumorigenesis. The phagocytic production of reactive radicals in response to foreign agents has also been proposed as a mechanism of initiation in cancers linked to infectious bacterial and viral agents such as human papilloma virus (HPV), hepatitis B and C viruses (HBV/HCV), H. pylori, and others(Morales-Sánchez & Fuentes-Pananá, 2014; Porta, Riboldi, & Sica, 2011).

These prior examples highlight the ability of stem cells to differentiate into cell types lost or damaged due to injury, but what happens when the stem cell populations themselves are depleted? Studies of the intestinal epithelium have identified two key mechanisms of recovery following depletion of CBCs, the highly proliferative stem cell population at the base of the intestinal crypt. CBCs have been characterized by high expression of the *leucine-rich repeat containing G-protein coupled receptor 5* gene (*Lgr5*) and are responsible for generating secretory and enterocyte precursors which terminally differentiate and divide upwards to populate the gut (Tetteh et al., 2016). Using a transgenic mouse model knock in of the diptheria toxin receptor under the *Lgr5* locus, Tian and colleagues showed that upon complete depletion of *Lgr5+* CBCs, *Bmi1* expressing quiescent stem cells at the +4 position underwent increased proliferation. Furthermore, lineage tracing showed that these *Bmi1* expressing cells gave rise to *Lgr5+* cells providing evidence that this population replenishes CBCs under injury and even normal conditions (Tian et al., 2011). An alternative mechanism to combat loss of CBCs is “dedifferentiation”, the process of a differentiated cell reverting to a less differentiated cell type within the same lineage (Tetteh, Farin, & Clevers, 2015a). Following depletion of CBCs by sub-lethal doses of radiation or ablation using the diptheria toxin receptor-transgenic mouse model, early secretory progenitors ($Dll1^{hi}$) and enterocyte progenitors (Alpi+) revert to stem cells (Tetteh et al., 2016; van Es et al., 2012). Tetteh and colleagues showed that upon depletion of both *Lgr5+* CBCs and *Bmi1* expressing +4 stem cell populations, *Alpi+* enterocyte progenitors dedifferentiate into stem cells but that this dedifferentiation potential is limited to the 2–3 day window before *Alpi+* cells have migrated out of the crypt and are no longer proliferative (Tetteh et al., 2016). This limited capacity for dedifferentiation highlights the intricacy of stem cell regulatory mechanisms and their dynamic interactions with extrinsic signals.

Stem cell plasticity – a continuum—The dedifferentiation potential of intestinal progenitors demonstrates another key characteristic of stem cells - plasticity. *Plasticity* is the ability of stem cells to delineate from homeostatic behavior and adopt an alternate cell fate in response to cellular and environmental needs such as injury or stress (Ge & Fuchs, 2018; Tetteh, Farin, & Clevers, 2015b) (Figure 2). The epithelial to mesenchymal transition (EMT) and its reverse, the mesenchymal to epithelial transition (MET), serve as prototypical

examples of plasticity, where cells undergo a phenotypic transition in response to environmental cues. EMT involves the modification of adhesive proteins and loss of apico-basal polarity allowing epithelial cells to acquire a more migratory and invasive phenotype with self-renewal capabilities (Scheel et al., 2011). During the gastrulation portion of embryogenesis, epithelial-like cells of the epiblast primary streak undergo EMT forming embryonic mesoderm which later gives rise to the primary mesenchyme (Kalluri & Weinberg, 2009). This process is tightly regulated by a combination of EMT transcription factors (Twist 1, Snail 1/2, Zeb 1/2;, and FOXC2), pro-inflammatory signals, cytokines, and signaling pathways Wnt and TGF-ß (Chaffer, San Juan, Lim, & Weinberg, 2016; Tsai, Donaher, Murphy, Chau, & Yang, 2012).

EMT and MET occur along a bidirectional continuum rather than a switch between two discrete states. Both immortalized human mammary epithelial cells induced for EMT and tumorigenic mammary cells have been found to co-express E-cadherin, an epithelial marker, and vimentin (VIM), a mesenchymal marker, indicating the presence of hybrid EMT/MET states and showcasing the gradient nature of these transitions (Mani et al., 2008; Yagasaki, Noguchi, Minami, & Earashi, 1996). Hybrid EMT/MET cells showed an increase in mammary stem cells compared to control (mammosphere assay) and the co-expression of E-cadherin and VIM in tumorigenic cells was associated with axillary metastases. These hybrid EMT/MET cells are also detectable in the normal mammary gland, and have a gene expression signature consistent with aggressive triple negative breast cancers (Colacino et al., 2018). “Partial EMT” has been observed in cancer epithelial cells, where partial transitions along the continuum are potentially more favorable to tumorigenesis than complete state shifts. The migratory characteristics acquired through EMT are favorable when carcinogenic cells leave the primary tumor site to metastasize, however upon arrival to a secondary tumor site, MET is necessary for cells to regain epithelial qualities in order to establish a metastatic colony and the outgrowths to support it (Chaffer et al., 2016). Indeed, carcinoma cells which have undergone a complete EMT program and lost all epithelial characteristics have been shown to be ineffective at proliferating and establishing metastases at secondary sites following dissemination. (Tran et al., 2014; Tsai et al., 2012). The necessity for EMT and plasticity in both embryogenesis and carcinogenesis highlights another striking similarity between these two processes.

The continuous nature of stemness observed during the EMT of early embryogenesis is also reflected in tissue specific differentiation. Studies using scRNA-seq have allowed us to observe these changes across the cellular landscape at single cell resolution (Giraddi et al., 2018; Macaulay et al., 2016; Nestorowa et al., 2016; Pal et al., 2017). These studies and others have pioneered a paradigm shift from the traditional view of differentiation as a unidirectional hierarchy of discrete stages to a dynamic and multi-directional continuum lacking clear boundaries between cell populations at varying stages of development (Y. Zhang, Gao, Xia, & Liu, 2018). This updated model emphasizes the intricate relationship between stem cells and environmental cues which guide the dynamic behavior of cells along the continuum (Fig1B). Using single cell transcriptomic expression to computationally order cells in a differentiation timeline (pseudotime), hematopoietic stem and progenitor cells isolated from mouse bone marrow and hematopoietic cells isolated from zebrafish kidneys both followed a continuous trajectory of differentiation directed by gradual changes in

transcriptomic expression (Macaulay et al., 2016; Nestorowa et al., 2016). Single cell RNA sequencing analyses of bulk mouse mammary epithelial cells sampled at varying stages of the life course followed a continuous sequential trajectory from most immature to most adult after single cell sequencing and mapping, rather than grouping in distinct cellular states (Giraddi et al., 2018; Pal et al., 2017). While the datasets from these two studies were generated separately and sampled different developmental time points (Giraddi: embryonic day 16, embryonic day 18, post-natal day 4, adult) (Pal: post-natal 2 weeks, post-natal 5 weeks, adult), the single cell mapping of both datasets clearly depict a gradual differentiation continuum rather than distinct clusters from the different developmental stages. These computational methods have shed major insight into the differences in transitional transcriptomic changes that varying cell types undergo as they progress through the differentiation landscape, opening the door for future research to use these transcriptional profiles to further study these intermediate states. The continuous nature of stemness and plasticity in development and in carcinogenesis has made it challenging to identify a clear and universal transcriptomic signature for stem cells, likely due to tissue specific differences in stem cell regulation. Significant progress, however, has been made in identifying markers for specific stem cell populations within tissues.

Epigenetic Regulation of Stem Cells—The coordinated gene expression necessary for stem cell maintenance, differentiation, cellular dedifferentiation, and transdifferentiation are established by epigenetic changes, including histone modifications, DNA methylation, and non-coding RNAs. Embryonic stem cells are characterized by an overall open chromatin state (Meshorer & Misteli, 2006). In these cells, key developmental genes, which are transcriptionally silent, are marked by bivalent chromatin domains, containing both the activating mark H3 lysine 4 methylation and the repressive mark H3 lysine 27 methylation (Bernstein et al., 2006). Acquisition of this bivalency state requires simultaneous histone modifications by the MLL2 and Polycomb complexes (G. Mas et al., 2018) Accompanying the open chromatin state is a reduced amount of global DNA methylation in naïve stem cells (Bibikova et al., 2006) but an increased amount of DNA hydroxymethylation specifically at the locations of bivalent chromatin domains (Pastor et al., 2011). As stem cells differentiate, these epigenetic patterns are altered, characterized by a gain of DNA methylation in pluripotency and germline-specific genes (Mohn et al., 2008) and an accumulation of transcriptionally inactive heterochromatin (Francastel, Schübeler, Martin, & Groudine, 2000). The entire 3D genomic architecture shifts during differentiation, as the interactions between different chromatin domains is altered to allow for the expression of concurrently regulated gene networks essential for the functioning of the differentiated cell (Dixon et al., 2015). Throughout the differentiation process, there is a simultaneous coordinated regulation of non-coding RNAs, which also dynamically shift to regulate the expression of key developmental pathways (Dinger et al., 2008). The epigenetic processes which regulate stem cell pluripotency and differentiation are tightly regulated and require the coordination of the various known mechanisms.

The dynamic epigenetic changes that occur during dedifferentiation in the induced pluripotency process (Takahashi et al., 2007) have provided important insights into developmental programming and epigenetic dysregulation in cancer. The temporal

epigenetic alterations that accompany embryonic stem cell differentiation are reversed during the induced pluripotency process, including DNA demethylation and acquisition of an open chromatin state (Takahashi & Yamanaka, 2015). Pluripotent stem cells are not, however, epigenetically equivalent to embryonic stem cells. Instead, these cells maintain an "epigenetic memory" of their tissue of origin, which survives through the reprogramming process (K. Kim et al., 2010). The epigenetic alterations during the reprogramming process have also shed light into CSC biology. Early genome-wide profiles of DNA methylation across multiple tumor types identified abundant enrichment of DNA methylation at sites required for differentiation in embryonic stem cells, marked by the Polycomb repressive complex, which helps lock tumor cells in a state of self-renewal (Widschwendter et al., 2007). Like embryonic stem cells, cancers are also typically characterized by a state of global DNA hypomethylation (Feinberg & Vogelstein, 1983). Extraction of epigenomic and transcriptomic feature sets from pluripotent stem cells and tumors using machine learning found that the most aggressive cancers have an epigenetic signature that resembles a stem cell (Malta et al., 2018). Further, metastasizing cells from breast cancer patients and mouse models of breast cancer in circulating tumor cell clusters are characterized by hypomethylation of key pluripotency related factors, such as OCT4, NANOG, and SOX2, reflecting a reacquisition of an embryonic stem-like phenotype in the most aggressive cancer cells (Gkountela et al., 2019). These results point to epigenetic alterations consistent with reprogramming to a stem cell state as a mechanistic driver of the cancer cell invasion and metastasis.

Identifying the Source of Cancer Stem Cells—While the evidence for a subpopulation of cancer cells with stem cell like properties is growing, the cell of origin for CSCs within the normal tissue hierarchy has not been formally defined. We hypothesize that there are three potential origins for CSCs. First, normal stem cells could acquire genetic and epigenetic changes that confer the ability to inappropriately undergo symmetric self-renewal and initiate tumorigenesis. A second possibility is that CSCs derive from lineage committed rapidly cycling progenitor cells that undergo mutations that reconfer stem-like properties. A final possibility is that a series of mutations in fully differentiated cells can lead to dedifferentiation to a tumorigenic stem state. For example, expression of a mutated form of the oncogene PIK3CA in luminal and basal mammary gland cells leads to the reacquisition of a multipotent state (Van Keymeulen et al., 2015). Conversely, transient expression of the stem cell pluripotency factors *Oct4, Sox2, Klf4,* and *Myc* in genetically modified mice, absent any other genetic mutation, is sufficient to induce cancers of epithelial origin in various tissues with an embryonic stem cell-like gene expression pattern (Ohnishi et al., 2014). As the body of research surrounding both normal and CSCs grows, evidence for multiple pathways of CSC generation is accumulating.

Identification and Assessment of Normal and Cancer Stem Cells—A key feature of normal stem cells that has proven essential to isolate and characterize these cells is the expression of specific surface and enzymatic markers of stemness. Early studies of the cellular hierarchy of the breast identified that the expression of the cell surface markers MUC-1- to ±/CD-10 ± to +/ESA+ isolated cells with the ability to develop colonies with both luminal and myoepithelial features (John Stingl, Eaves, Kuusk, & Emerman, 1998). A

follow-up study identified that normal bipotent mammary progenitors are enriched in a cellular subfraction expressing both CD49f (α6 integrin) and EpCAM (J. Stingl, Eaves, Zandieh, & Emerman, 2001). Others have identified that normal bipotent mammary stem cells are further enriched in the CD44+/CD24-fraction of CD49f+/EpCAM+ cells (Ghebeh et al., 2013). LGR5 is a well characterized marker of normal and cancer stem cells in multiple tissues (Leung, Tan, & Barker, 2018), and methods were recently published for the antibody-based isolation of LGR5-expressing normal and cancerous cells from human primary intestine and colonic organoids (Dame, Attili, McClintock, Dedhia, Ouillette, et al., 2018). In addition to stem cell enriching cell surface protein markers, enzymatic markers of stemness have also been identified. Normal breast stem and progenitor cells express high levels of aldehyde dehydrogenase 1 (ALDH1) (Ginestier et al., 2007). Similar results for enrichment of ALDH1 expression in stem cells were also found in normal and cancerous colon (E. H. Huang et al., 2009), head and neck cancers (Clay et al., 2010), and pancreatic cancer (M. P. Kim et al., 2011). ALDH1 expressing cells can be identified using the non-immunological Aldefluor assay, where the substrate, Bodipy-aminoacetaldehyde, is converted intercellularly to fluorescent Bodipy-aminoacetate. Stem cells can also be isolated by exploiting their increased expression of ATP-binding cassette drug transporters (Bunting, 2002). By staining cells with Hoechst, a DNA-binding dye that is effluxed by ATP-binding cassette transporters, one can discriminate populations of cells that are high and low Hoechst staining, with the low-Hoechst stained, stem cell enriched, fraction termed the “side population”.

Model organisms genetically engineered to express fluorescent reporters of stem cell regulators have allowed for in depth analyses of stem cells in development, tissue homeostasis, and disease. In particular, genetically engineered mice have been widely used to trace stem cell differentiation and understand the impact of alterations to the stem cell niche. In a seminal study, Barker and colleagues developed the Lgr5-EGFP-IRES-creERT2 knock-in mouse line, which allows for immunofluorescent identification of Lgr5+ stem cells and was used to provide substantial evidence of the essential role these cells have in generating the intestinal epithelium (N Barker et al., 2007). These mice have been used to assess the function of Lgr5+ cells in development and tissue homeostasis across a range of organ systems including mammary gland (Plaks et al., 2013), stomach (Nick Barker et al., 2010), liver (Planas-Paz et al., 2016), lung (Lee et al., 2017), prostate (B. E. Wang et al., 2015), and ovary (Ng et al., 2014). Directed knockout of the Wnt pathway activator APC in these intestinal Lgr5+ cells led to the rapid development of adenomas, suggesting that these cells are a likely cell of origin in APC driven colon cancers (Nick Barker et al., 2009). Directed knockout of the tumor suppressor gene p53 in Lgr5+ cells in mouse treated with a DNA damaging agent, azoxymethane, and an inflammation inducing agent, dextran sodium sulfate, showed that stem cell specific deletion of p53 significantly increases colon tumor size and incidence in this inflammation and DNA-damage driven model (Davidson et al., 2015). Additionally, the differentiation hierarchy of the hematopoietic system is very well characterized, and transgenic mice with various reporters for cell types across this hierarchy have been developed (Vacaru, Vitale, Nieves, & Baron, 2014). These models hold significant promise to provide fundamental understanding of how environmental factors perturb stem cell biology *en route* to cancer formation. Specifically, they allow for the interrogation of

stem cells *in situ* through microscopy or flow cytometry, are amenable to further genetic manipulation to either drive Lgr5 reporter expression in specific tissues or knock out cancer-related genes, and allow for the assessment of stem cell function in the presence of the entire stem cell niche.

A number of functional assays for the identification and classification of both normal and cancer stem cells have been established. The first series of experiments to identify the presence of tumor initiating cells utilized a transplantation assay of human acute myeloid leukemia cells into severe combined immunodeficient (SCID) mice (Lapidot et al., 1994). The SCID mice were examined for the presence of human leukemia cells, with limiting dilution experiments identifying that approximately 1 in 250,000 cells have the ability to engraft, with the cells most likely to engraft possessing the CD34+CD38-hematopoietic progenitor signature. In the human breast, cancer stem cells were first identified by injecting single cell suspensions of dissociated human breast tumor tissue into NOD/SCID mice (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). Tumor cells were sorted based on cell surface markers (CD44, CD24, EpCAM) and injected into the mammary fat pads of mice at limiting dilutions, revealing that as few as 200 ESA+/CD44+/CD24-cells were consistently able to form tumors, while injection with 20,000 CD44+/CD24+ cells failed to grow tumors (Al-Hajj et al., 2003). Furthermore, the ESA+CD44+CD24-cell fractions were able to recapitulate the original tumor phenotype after serial transplantations, showing that these cells possess the ability to both proliferate and differentiate into the different cell types that comprised the original tumor. In general, the serial reimplantation assay is considered the “gold standard” assay in the field to identify cancer stem cells from human tumors, although the assay has a number of potential weaknesses, including the injection of dissociated single cell suspensions rather than complete microenvironments and the lack of a competent immune system in the host animal (Rycaj & Tang, 2015).

In addition to the transplantation assays described above, a number of other assays have been utilized to enrich and characterize normal and cancer stem cells. Neural stem cells were first discovered to grow in anchorage independent, serum free culture conditions forming free floating spheroids of neural cells termed neurospheres (Reynolds & Weiss, 1996). These culture conditions were later adapted for mammary tissue, where both tumor and normal breast stem/progenitor cells were found to propagate under these conditions, termed mammosphere formation conditions (Dontu et al., 2003). Spheroid conditions have now been applied to study stemness and 3D structure in a range of different normal and tumor types (Colacino, 2016). Tumorsphere formation is useful for characterizing three key aspects of stem cell biology: (1) proliferation potential; (2) the ability to self-renew; and (3) the ability to differentiate into downstream progeny. Since each tumorsphere is initiated by a single cell, proliferation capacity can be assessed by tumorsphere size. Tumorspheres can then be serially passaged to assess stem cell self-renewal capacity over time. Finally, tumorspheres can be stained for known markers of lineage differentiation, or plated into differentiating culture conditions to assay the potency of the stem cell population.

Human organoid cultures afford advantages over spheroid models as they allow for the long-term propagation of stem cell enriched patient derived samples in a physiologically relevant extracellular matrix. By varying the composition of the matrix, different properties relevant

to cancer stem cells can be altered, including proliferation, invasion, and dissemination (Shamir et al., 2012). Biobanks of normal and cancerous patient derived organoids have been established for breast (Sachs et al., 2018), colon (Dame, Attili, McClintock, Dedhia, Ouilette, et al., 2018; Fujii et al., 2016; van Sluis et al., 2015), and prostate (Beshiri et al., 2018; Gao et al., 2014). These organoid biobanks are comprised of tissues which span the spectrum from normal, to early stage, to advanced cancers, which provides unique resources to understand alterations in stem cell biology through carcinogenesis. New experimental techniques are being developed to improve the long term survival, cellular diversity, and physiological relevance of these 3D culture systems (Fujii et al., 2018; Neal et al., 2018). While experimental analyses of environmental effects on stem cell biology in organoid systems are still in their infancy, these models have been used to assess the impact of dietary fat, essential nutrients, and toxic heavy metals on stem cell regulation relevant to tumorigenesis (Beyaz et al., 2016; McClintock et al., 2018; Rocco et al., 2018).

The Environment and Cancer – Are Stem Cells the Key?—While genetic risk factors for cancer have been well characterized and described, an estimated 70–90% of lifetime cancer risk is estimated to derive from exposures to extrinsic factors (Wu, Powers, Zhu, & Hannun, 2016). Similar etiologic contributions of environmental risk factors have been estimated for other chronic diseases (Rappaport, 2011). Although there is debate within the literature, high profile studies have also identified that the number of stem cells, and stem cell divisions, in a tissue is a strong predictor of the probability of developing cancer in that tissue (Albini, Cavuto, Apolone, & Noonan, 2015; Tomasetti, Li, & Vogelstein, 2017; Tomasetti & Vogelstein, 2015). The ‘bad luck’ theory of carcinogenesis by Tomasetti and Vogelstein is still controversial due to potential limitations in the study including the cancers studied and the fact that association does not mean causation (Albini et al., 2015). Additionally, emerging evidence points to the number or proportion of stem cells in a given normal tissue between different individuals is highly variable (Colacino et al., 2018; Kumar et al., 2018; Nakshatri, Anjanappa, & Bhat-Nakshatri, 2015). Studies of patient tissues have identified some of the predictors of tissue stem cell number. Women who inherit BRCA1 mutations have a higher proportion of stem cells in their non-cancerous breast tissue (Honeth et al., 2015; Merajver et al., 2008). Obesity is associated with an increase in the number of functional mammary epithelial progenitor cells in breast tissue from healthy women (Chamberlin, D’Amato, & Arendt, 2017). Quantifying the impacts of both intrinsic and extrinsic risk factors on stem cell function will be essential to understanding why people get cancer.

A growing body of experimental literature is showing that environmental exposures during development can have lasting impacts on stem cells, with important implications for cancer risk. The concept of windows of susceptibility proposes that exposures during critical time points, such as early life development, can result in life-long effects due to the vulnerability of developing organ systems (Birnbaum & Fenton, 2003). Young children and developing fetuses are especially vulnerable due to incomplete or the lack of development of protective mechanisms such as DNA repair, immune system function, detoxifying enzymes and liver metabolism, and the blood/brain barrier (Schug, Janesick, Blumberg, & Heindel, 2011). The *in utero* period of development is a period of heightened susceptibility due to the rapid

metabolism and proliferation of developing organs. Embryonic development is a stage of extreme vulnerability to environmental exposures which may signal and interfere with epigenetic programming, which has been shown to persist in daughter cells and even trans-generationally (Leslie, 2013; Skinner, Manikkam, & Guerrero-Bosagna, 2010; Zeybel et al., 2012). Impaired organ development early on in life has been shown to alter development at critical downstream time points such as puberty and pregnancy (Rudel, Fenton, Ackerman, Euling, & Makris, 2011). Understanding that timing is key in the damaging potential of environmental exposures on stem cell populations will ultimately lead to efforts to prevent adverse exposures and the resultant effects in the most vulnerable populations—pregnant women, developing fetuses, and young children.

2. Exposures and Cancer Stem Cells

Evidence is building that stem cells are a key target for environmental exposures relative to the formation of cancers. Here, we will review the evidence linking environmental exposures and dysregulation of stem cell biology across known chemical, biological, and physical hazards.

CHEMICAL EXPOSURES

Metals

Arsenic: Chronic arsenic exposure leads to the acquisition of cancer stem cell-like characteristics in immortalized human cells from a diverse set of tissues, including lung (Q. Chang, Chen, Thakur, Lu, & Chen, 2014; Person et al., 2015), prostate (Tokar et al., 2010), bladder (Ooki et al., 2018), and mammary gland (Xu, Tokar, & Waalkes, 2014). Consistent molecular alterations were observed across the various cell types, including increased matrix metalloproteinase secretion, colony formation, invasion, and expression of stem cell markers. Functional analyses to define the CSC-associated mechanisms in the prostate epithelial cells transformed following an 18 week exposure to 5μM inorganic arsenic found that forced overexpression of *miR-143*, a miRNA whose expression was downregulated with arsenic transformation, mitigated the CSC phenotype, including decreased MMP-2 and MMP-9 secretion, decreased cell proliferation, and decreased apoptotic resistance (Ngalame, Makia, Waalkes, & Tokar, 2016). *miR-143* is an important regulator of bone metastases in prostate cancer, and forced expression can reduce prostate cancer sphere formation and decreased expression of stemness markers, including CD133, CD44, Oct4, and MYC (S. Huang et al., 2012). DNA methylation profiling of these arsenite transformed cells identified a significant enrichment of hypermethylation in domains occupied by histone H3 lysine 27 methylation, a silencing pattern associated with cancer (Severson, Tokar, Vrba, Waalkes, & Futscher, 2012). The same arsenic transformed prostate epithelial cells also increased their secretion of exosomes 700% relative to the parental cell line (Ngalame, Luz, Makia, & Tokar, 2018). Profiling the exosomes from the arsenic transformed line identified that the cargo was made up of oncogene and inflammatory mRNA transcripts as well as oncogenesis-associated miRNAs. Treatment of non-transformed prostate epithelial stem cells with exosome-containing conditioned media isolated from the arsenic transformed cells also induced a cancer stem cell-like phenotype in the non-transformed cells, characterized by increased matrix metalloproteinase secretion and a phenotype reflecting an epithelial-

mesenchymal transition (Ngalame et al., 2018). These findings highlight an alternative mechanism of environmental carcinogenesis, by which transformed cancer stem cells can induce an oncogenic phenotype in non-transformed stem cells through an exosome-mediated mechanism.

Additionally, arsenic exposure *in vivo*, particularly early in life, can induce carcinogenesis later in life – consistent with the developmental origins of adult cancer hypothesis (Tokar, Qu, & Waalkes, 2011). Offspring of pregnant C3H mice exposed to 42.5 and 85 ppm of sodium arsenite in drinking water developed an increased number of tumors at a range of sites, including liver tumors in both sexes, adrenal tumors in male offspring, and ovarian and lung tumors in female offspring (Waalkes, Ward, Liu, & Diwan, 2003). Importantly, the increased rate of tumor incidence in the exposed offspring had a dose-dependent response, with higher tumor formation rates in the 85ppm exposed animals (Waalkes et al., 2003). Similar findings have been reported for early-life arsenic exposure and risk of lung and bladder cancer in a population of highly exposed individuals in Chile (Steinmaus et al., 2014). A dose-dependent response was observed, with individuals who were exposed to early life arsenic water concentrations >800 μg/L having a 5.24-fold increased odds for lung cancer, and 8.11-fold increased odds for bladder cancer, than individuals exposed to less than 110μg arsenic/L in their water (Steinmaus et al., 2014). As these early life exposures to arsenic induce a tumor spectrum resembling those caused by estrogenic compound exposure, the effects of *in utero* arsenic exposure were specifically evaluated for alterations in estrogen signaling (Waalkes et al., 2004). Analysis of estrogen signaling in liver samples from the 85ppm and control C3H mice from the (Waalkes et al., 2003) study, along with liver samples isolated from patients in an area of Guizhou, China with high levels of endemic arsenic and control liver samples from University of North Carolina patients, found that in both mice and humans, arsenic exposure was associated with an increased expression in estrogen receptor alpha (ERα) (Waalkes et al., 2003). Provided further validation of the hypothesis that early life arsenic exposure can have estrogenic effects, which may influence later life cancer risk, are investigations of early life arsenic exposure and dysregulated mammary morphogenesis. *In utero* exposure to 5 μg arsenite /kg/bw by intraperitoneal injection on days 12 and 17 of gestation led to an increase in the number of mammosphere forming cells at postnatal day 5 and an increase in ERα expression in the post-pubertal mammary gland (Parodi et al., 2015). In aggregate, these results show that developmental arsenic exposures can influence stem cell differentiation trajectories, hormonal signaling, and cancer risk across a range of tissue sites.

Intriguingly, arsenic is also under active investigation as a cancer therapy, through targeting cancer stem cells. Arsenic trioxide is an established treatment for acute promyelocytic leukemia (Antman, 2004), and is currently being tested as a treatment for a range of solid tumor types. Preclinical research in MHCC97H and MHCC97L hepatocellular carcinoma cell lines identified that 10 μM arsenic trioxide treatment for 72 hours reduced the proportion of cancer stem-like cells through the expression of miR-491, a regulator of TGFβ signaling (Y. Li et al., 2015). In lung cancer, 1–4 μM arsenic trioxide treatment reduced the tumorsphere formation rates of both NCI-H460 non-small cell and NCI-H446 small cell lung cancer cell lines in a dose-dependent manner (K.-J. Chang, Yang, Zheng, Li, & Nie, 2016). These functional alterations were accompanied by a dose-dependent downregulation

of Gli1 expression, an essential transcription factor in the developmental Hedgehog pathway, as well as downregulation of pluripotency factors Sox2 and Oct4 (K.-J. Chang et al., 2016). In an unbiased high throughput drug screen to target glioblastoma, arsenic trioxide was identified from a panel of 650 drugs as a potent inhibitor of the proneural stem cell populations in patient derived glioblastoma cell lines (Bell et al., 2018). The discrepancy of arsenic's action as a carcinogen and cancer therapy was assessed in a transformed human keratinocyte cell line, HSC5 cells, exposed to 1–5μM arsenic, a concentration relevant to the groundwater contamination levels in Bangladesh (Thang, Yajima, Kumasaka, & Kato, 2014). At 3 μM treatment with arsenic, these cells simultaneously exhibited increased invasive capacity through upregulation of MT1-MMP as well as increased apoptosis through increased p21 expression (Thang et al., 2014).

Cadmium: Cadmium is a known human carcinogen with an established link to lung cancer through occupational health studies (Waalkes, 2003). The major routes of exposure are industrial settings, dietary intake of cadmium containing foods, and tobacco smoking. The effects of cadmium on cancer and stem cell related pathways have been investigated in model systems representing multiple organ types. Human ductal pancreatic epithelial cells exposed to low dose (1μM) cadmium for 29 weeks *in vitro* acquired multiple characteristics associated with cancer stem cells, including increased anchorage independent growth as spheroids, increased expression of the pancreatic cancer marker S100p, increased invasive capacity, and increased expression of known stem cell factors, including *OCT4* and *CD44* (Qu, Tokar, Kim, Bell, & Waalkes, 2012). Repeated cadmium exposure of immortalized human non-tumorigenic prostate cells (pRNS-1–1 cells) caused increased anchorage independent growth and tumor formation when implanted into immunosuppressed mice (Nakamura et al., 2002). Non-contact co-culture of human prostate epithelial cells transformed following an 8 week exposure to 10μM cadmium and normal prostate stem cells led to the normal prostate stem cells acquiring invasive properties, including 3D branching growth in matrigel, accompanied by an overexpression of a number of factors associated with EMT, including VIM, SNAIL1, and TWIST1 (Xu et al., 2013). An investigation into the mechanistic drivers of cadmium induced stemness and oncogenic transformation in these cadmium transformed prostate cells identified that KRAS overexpression is likely an important driver of the phenotype, as KRAS knockdown mitigated colony formation, cell survival, and expression of MMP-2 (Ngalame, Waalkes, & Tokar, 2016).

There is conflicting evidence linking cadmium exposure, altered stemness, and breast cancer. Multiple case-control studies have identified urinary cadmium concentrations are elevated in breast cancer cases relative to controls (Gallagher, Chen, & Kovach, 2010; McElroy, Shafer, Trentham-Dietz, Hampton, & Newcomb, 2006; Nagata et al., 2013; Strumylaite et al., 2014). Follow up studies of breast cancer risk associated with estimated dietary cadmium intake from cohort studies have largely been equivocal (Van Maele-Fabry, Lombaert, & Lison, 2016). There is building experimental evidence, however, that cadmium exposure can dysregulate biological pathways and processes associated with development and stemness. In Sprague-Dawley rats, *in utero* exposure to 0.5 μg/kg cadmium on days 12 and 17 of gestation has an estrogen-mimicking effect on the developing mammary gland, leading to an increased proportion of terminal end buds (Johnson et al., 2003; Parodi et al.,

2017), structures enriched for mammary stem cells (Kenney, Smith, Lawrence, Barrett, & Salomon, 2001). These same results were not observed at a higher dose, 5 μg/kg cadmium, although the epithelial area of the mammary gland was increased in these rats (Johnson et al., 2003), suggesting that there may be a non-monotonic dose response relative to cadmium exposure on mammary terminal end buds. In a follow-up study, Sprague-Dawley rats exposed to 5 μg/kg bw on days 12 and 17 of gestation had an increase in mammary gland branching, as well as an increase in the number of mammosphere forming stem cells and overexpression of ERα (Parodi et al., 2017). Dietary *in utero e*xposure of Sprague-Dawley rats to 75 of 150μg Cd/kg of food also led to increases in offspring mammary gland terminal end buds at the 75 μg Cd/kg dose, but did not alter 7, 12-dimethylbenz(a)anthracene-induced mammary cancer (Davis, Khan, Martin, & Hilakivi-Clarke, 2013) Experimental analyses of cadmium exposed immortalized or transformed breast epithelial cell lines identified the acquisition of a mammary stem cell-like mesenchymal phenotype consistent with EMT (Benbrahim-Tallaa et al., 2009; Wei, Shan, & Shaikh, 2018). Conversely, analyses of primary normal human mammary cells, isolated from voluntary reduction mammoplasties, treated with 2.5μM cadmium showed downregulation of EMT-associated genes, including *VIM* and *ZEB1*, and decreased invasive capacity in organoid forming conditions (Rocco et al., 2018). Primiparous NMRI mice exposed to subcutaneous cadmium injections (5, 100, and 2000 μg Cd/kg body weight) on lactational day 8–10 had stunted mammary gland development, reflected by dose-dependent decreases in β-Casein gene expression and alterations in mammary gland morphology (Davis et al., 2013; Öhrvik, Yoshioka, Oskarsson, & Tallkvist, 2006). Taken together, these results suggest that cadmium may have divergent effects on mammary stem cells depending on the window of susceptibility of exposure as well as the stage of carcinogenesis.

Hexavalent Chromium: Hexavalent chromium is a known human carcinogen, which has been linked to lung cancer in occupational exposure settings (Gibb, Lees, Pinsky, & Rooney, 2000). Emerging evidence points to dysregulating stem cell associated pathways as a potential mechanism of hexavalent chromium associated carcinogenesis. Exposure to the non-tumorigenic lung epithelial cell line BEAS-2B to a continuous 1μM hexavalent chromium exposure led to increased cellular proliferation, anchorage independent growth, and tumor formation capacity in immunosuppressed mice (He et al., 2013). The hexavalent chromium transformed cells were also characterized by a 35-fold decreased expression of miR-143, an important regulator of IGF-IR/IRS1 signaling, as well as tumor growth and angiogenesis (He et al., 2013). BEAS-2B cells transformed following a 20 or 40 week treatment with 250nM hexavalent chromium revealed significant epigenetic alterations, including increases in repressive H3K9me2 and H3K27me3 chromatin marks and in the histone methyltransferases G9a, SUV39H1, EZH2, and GLP (Z. Wang et al., 2018). Intriguingly, shRNA knockdown of G9a, SUV39H1, and EZH2 in non-transformed BEAS-2B cells exposed to 125nM hexavalent chromium for 25 weeks significantly decreased the anchorage independent growth of these cells relative to control shRNA BEAS-2B cells, suggesting that upregulation of these histone methyltransferases may be essential for hexavalent chromium's promotion of stemness (Z. Wang et al., 2018). Another analysis of BEAS-2B cells transformed following a 3 month continuous exposure to 100nM hexavalent chromium identified a subpopulation of cancer stem cell-like cells, isolated

following anchorage independent growth in the secondary spheroid assay (Dai et al., 2017). These chromium-induced cancer stem cells had significantly increased tumor initiation capacity in immunocompromised mice, as well as increased expression of the stem cell regulator Notch 1 (Dai et al., 2017). This cancer stem cell enriched population also displayed reduced sensitivity to cisplatin, reduced capacity for the generation of reactive oxygen species, and increased rates of glycolysis, which was linked to loss of expression of FBP1, a rate-limiting enzyme in gluconeogenesis (Dai et al., 2017). In an immortalized human kidney cell line, HK-2, chromium exposure (0.5 – 2μM) induced a dose-dependent increase in the expression of mesenchymal markers Vimentin and SMA, as well as an increase in the stem cell markers Nanog and CD133 (W. J. Li, Yang, Chow, & Kuo, 2016). These changes were linked to a decrease in dihydrodiol dehydrogenase expression, providing further evidence that hexavalent chromium's effects on stemness associated pathways may be driven by metabolic alterations (W. J. Li et al., 2016). In aggregate, these studies suggest that both epigenetic alterations and metabolic reprogramming may be key drivers in chromium induced carcinogenesis, and point towards understanding the intersection between these biological processes as an important area for future research.

Tobacco Smoke—Tobacco smoking is associated with death from multiple cancers, including lung, colorectal, stomach and liver, and tobacco smoking is estimated to cause approximately 10 million deaths per year (Proctor, 2001; Torre et al., 2015). The genotoxic effects of exposure to the complex mixture of chemicals in tobacco smoke are well characterized (DeMarini, 2004). There is emerging evidence that tobacco smoke and nicotine can modify stem cell associated pathways relevant for carcinogenesis across multiple organ systems. In mice exposed to tobacco smoke with total particulate matter of 85 mg/m^3 for 12 months using a smoking apparatus, histopathological analyses of the liver tissues identified changes consistent with an EMT and increased Oct4 and Nanog expression, which was linked to an increase in IL-33 expression and phosphorylated p38 (Xie et al., 2019). Co-exposure of mice to tobacco smoke and the p38 inhibitor SB203580 (1 mg/kg body weight) for 12 weeks attenuated the tobacco smoke-induced upregulation of stemness factors CD133, Nanog, and Oct4 (Xie et al., 2019). Exposure of non-tumorigenic (MFC10A and MCF12A) and tumorigenic (MCF7) breast cell lines to tobacco smoke extract for 72 weeks or cigarette smoke condensate for 40 weeks led to dose-dependent acquisition of an EMT-like phenotype, increased anchorage independent growth, and increased metastatic dissemination when implanted into immunocompromised mice (Di Cello et al., 2013). Exposure of two head and neck squamous carcinoma cell lines (UMSCC10B and HN-1) to 0.1, 1, or 3mM nicotine for 6 weeks similarly caused a dose-dependent increase in the expression of EMT-associated markers Snail, Twist, and Vimentin (Yu et al., 2012). These changes were accompanied by the induction of stem cell markers Oct4, Nanog, CD44, and BMI-1, although the highest induction of these factors was observed at the 1mM nicotine exposure(Yu et al., 2012). In the lung, long term exposure of immortalized human bronchial epithelial cells to 2% cigarette smoke extract for 25 weeks caused an increase in expression of cancer stem cell associated genes, including CD133 and ALDH1 and an increase in spheroid formation and upregulation of Wnt pathway members p-GSK3β, β-Catenin, and C-Myc (J. Wang et al., 2018). Nicotine (2μM) or e-cigarette extract exposure for around 24 hours in non-small cell lung cancer cell lines A549 and

H1650 caused upregulation of Sox2 and an EMT-like phenotype through activation of nicotinic acetylcholine receptors, Yap1, and E2F1 (Schaal, Bora-Singhal, Kumar, & Chellappan, 2018). Early life tobacco smoke exposure through second hand smoke has also been associated with an increased risk of later life lung cancer, and that this was modified by a polymorphism in the dopamine receptor gene *DRD1* (Ryan et al., 2013). Developmental tobacco smoke exposure is also known to cause very consistent DNA methylation alterations in blood (Joubert et al., 2016), which persist into adulthood (Richmond, Suderman, Langdon, Relton, & Smith, 2018). These same DNA methylation alterations were also enriched in lung cancer tissues isolated from smokers from the Cancer Genome Atlas (Bakulski, Dou, Lin, London, & Colacino, 2019). Finally, exposure of immortalized human urothelial cells to cigarette smoke extract for 20 weeks led to an increase in anchorage independent growth, an increase in growth in immunocompromised mice, increased expression of EMT markers and upregulation of Wnt/β-catenin signaling (Liang et al., 2017). Treatment with curcumin, a polyphenol derived from turmeric, led to a downregulation of Wnt signaling and a reversal of the EMT phenotype associated with the cigarette smoke extract (Liang et al., 2017). Curcumin has been shown to have similar stem cell targeting effects in other tissues as well (Colacino, McDermott, Sartor, Wicha, & Rozek, 2016; Kakarala et al., 2010; Zang, Liu, Shi, & Qiao, 2014), suggesting that this compound may have value as a stem cell differentiation agent for cancer prevention and treatment.

Endocrine disrupting chemicals (EDCs)—Numerous epidemiological studies have previously connected endocrine disruption with an increase in cell stemness. In a study assessing the epigenetic effects of hormonal exposures, 120 (0.5%) of all CpG sites analyzed were hypermethylated in epithelial cells exposed to estrogen (Rodriguez et al., 2008). Of these 120 loci, 111 were methylated in the transcription start sites of genes (Rodriguez et al., 2008). In this same study 23% of the methylated targets were also Polycomb group proteins which direct pluripotency of stem and progenitor cells (Rodriguez et al., 2008). Key developmental regulators appeared to also be epigenetically targeted by estrogen since 8 of the loci with hypermethylation were also tumor suppressor genes that are down-regulated in cancers (EGR2, FANCF, MXI1, PTPRG, RPRM, RUNX3, TFAP2C, and WNT5A) (Rodriguez et al., 2008). The results highlight the impact of estrogen signaling, and its potential for dysregulation by EDCs, in stem cell programming.

Diethylstilbestrol: EDCs are becoming an increasing concern to human health, particularly during susceptible stages of development. A substantial amount of our understanding about the effects of developmental exposure to hormone mimicking compounds and later life cancer risk comes from the cohort of women who were exposed to diethylstilbestrol (DES) *in utero*. DES was prescribed to women considered at elevated risk of having a miscarriage starting in the 1940s (Hilakivi-Clarke, 2014). By the 1970s, physicians were observing increased incidence of rare adenocarcinomas of the vagina in women who were exposed to DES *in utero* (Herbst, Ulfelder, & Poskanzer, 1971). Follow up studies of these “DES daughters” have also revealed an increased risk of breast cancer (Troisi et al., 2007). Mechanistic studies have found that a 3-week exposure to normal human mammary stem cells to 70nmol/L DES led to widescale epigenetic remodeling, including miRNA alterations, in epithelial progeny (Hsu et al., 2009). Downregulation of *miR-9–3*, an

important regulator of p53-related apoptosis, was accompanied by an increase in the repressive H3K27me3 mark (Hsu et al., 2009). CD1 mice exposed *in utero* to maternal intraperitoneal injections of 10μg/kg DES during days 9–26 of gestation had an approximately 2-fold upregulation of expression of EZH2, the histone methyltransferase which catalyzes the addition of methyl marks to H3K27 in the mammary gland tissue (Doherty, Bromer, Zhou, Aldad, & Taylor, 2010). Exposure to DES has also been shown to influence the size of the stem cell pool in a model of uterine fibroids (A. Mas et al., 2017). Neonatal female Eker rats exposed to a subcutaneous 10μg dose of DES on days 10, 11, and 12 after birth had an approximately 15-fold increase in the number of $Stro1^{+}/CD44^{+}$ uterine myometrial stem cells, and the increase in stem cell number persisted (although was attenuated) through 5 months of age (A. Mas et al., 2017). Increased epigenetic silencing of developmental mediators leading to an expanded normal stem cell pool caused by early life exposure to DES is likely a key mechanism linking DES exposure to later life cancer risk.

Bisphenol A: Bisphenol-A (BPA) is a ubiquitous plasticizer and xeno-estrogen detected in >95% of the US population (Calafat, Ye, Wong, Reidy, & Needham, 2008; Hindman et al., 2017). A study of in utero exposure to BPA in CD-1 mice showed that the offspring of pregnant mice exposed to 25μg/kg BPA during E8.5-E18.5 by intraperitoneal (IP) injection exhibited significant mammary defects compared to those dosed with vehicle control (sesame oil) (Hindman et al., 2017). The most substantial mammary defects occurred during E12.5–16.5, the time during which the developing mammary epithelial bud is completely surrounded by the ERα positive stroma. The mammary epithelial bud itself has been shown to be ERα negative, thus this study highlighted the importance of the mesenchymal stroma in conjunction with ERα in mammary morphogenesis and the potency of exposure during critical time points. In an *in vitro* study, 10nM exposure to BPA on day 5 of culture induced both mRNA and protein expression of ALDH1 in MCF-7 ER-positive cancer cells compared to ethanol-treated controls (Lillo, Nichols, Seagroves, Miranda-Carboni, & Krum, 2017). Another observation was an increase in size of MCF-7 mammospheres and mammospheres grown from ER+ breast tumor patient derived xenografts dosed with 10nM BPA compared to EtOH controls which was accompanied by an increase in histone H3 trimethylation (Doherty et al., 2010). MCF-7 mammospheres treated with 10nM BPA also showed significant mRNA and protein induction of the *SOX2* transcription factor, known to be involved in pluripotency and self-renewal (Lillo et al., 2017). Conversely, 10nM BPA treated MDA-MB-231 triple negative breast cancer cells, which do not express the estrogen receptor (ER), showed no increase in mammosphere size and no significant induction of *SOX2* (Lillo et al., 2017). *In utero* exposure of CD-1 mice to 10μg/kg BPA through IP injection of dams during days 9–26 of gestation led to increased expression of the Polycomb transcription factor member EZH2 (Doherty et al., 2010). Increased EZH2 expression has been linked to increased aggressiveness and tumor proliferation in human breast carcinomas as well as impaired DNA repair in MCF10A mammary epithelial cells (Collett et al., 2006; Zeidler et al., 2005) This body of work highly implicates regulation of cancer stem cell activity through *SOX2,* estrogen, and epigenetic pathways. In addition to the breast, BPA has also been implicated in acting on estrogen signaling pathways in the prostate. Using primary epithelial prostate cells from healthy donors, Prins and colleagues showed that both 1nM estrogen (E2) and 10nM BPA treatment resulted in an increase in size and number of 3D-

prostaspheres, showing that prostate cells are E2-sensitive and that the estrogen pathway is a target of BPA (Prins et al., 2014). These BPA treated prostaspheres also showed a significant increase in gene expression of known stemness genes *TBX3* and *Nanog*. Furthermore, they showed that in an *in vivo* model where epithelial stem-like progenitor cells from healthy donors are grafted into rats, oral exposure to 100 or 250 μg/kg body weight of BPA during development resulted in an increase in malignant lesions from 13% in normal grafts to 36% and 33% in BPA treated grafts. In a later study, Prins and colleagues exposed male rats to vehicle control, positive control ethinyleestradiol (EE), or BPA from gestational day 6 up to 6 months of age after which prostaspheres were cultured for 3 generations (21 days) without exposure (Prins et al., 2018). They found that rats exposed to 0.5μg EE/kg-BW and 2.5μg BPA/kg-BW showed a significant increase in third generation prostaspheres compared to the vehicle control. Additionally, they found that 0.5μg EE/kg-BW, 25μg BPA/kg-BW, and 250 μg BPA/kg-BW exposures resulted in a significant increase in large prostaspheres (>80μm) compared to vehicle control, suggesting an increase in proliferative capability of these stem cells. Together these findings demonstrate that BPA can directly act on estrogenic pathways in stem-progenitor cells, increase self-renewal and stem capabilities through downstream actions, and increase later life risk of hormonal carcinogenesis in response to developmental exposure.

Bisphenol Analogues: Although BPA is being phased out of consumer products, there is building evidence that its analogues BPB, BPF, BPAF and BPS, which are similar in structure, also exhibit endocrine disrupting potency through estrogenic and oxidative stress pathways. In fact, in MCF-7 human breast cancer cells, BPAF was found to have a binding affinity for ERα 20 times greater than BPA (Kitamura et al., 2005). *In vitro* treatment of rat sperm with BPA, BPB, BPAF, and BPAF showed a significant increase in superoxide dismutase and reactive oxygen species (ROS) compared to controls at 100μg/L (Ullah, Pirzada, Jahan, Ullah, & Khan, 2019). Analogues produced equivalent if not greater increases in superoxide dismutase expression and ROS compared to BPA, indicating that they are potent inducers of oxidative stress and likely cause toxicity through mechanisms similar to BPA. CD-1 mice exposed to BPAF (0.05, 0.5, 5 mg/kg) and BPS (0.05, 0.5, 5 mg/kg) *in utero* during gestation days 10–17 showed significant alterations in mammary gland development compared to vehicle controls between PND 20–56. (Tucker, Hayes Bouknight, Brar, Kissling, & Fenton, 2018). Acute gestational exposure to BPAF and BPF resulted in early pubertal alterations in the mammary gland including an increase in terminal end bud number and increased branching density in a dose dependent manner. Abnormalities in mammary morphology persisted into late adulthood, where BPAF and BPS exposed female offspring exhibited undifferentiated end ducts and significantly higher incidence of non-neoplastic lesions and spontaneous adenocarcinomas. Bisphenol analog exposed groups also exhibited significantly higher incidences of inflammation, measured by lymphocyte, plasma cells, and macrophage infiltrates.

Gonadotropin releasing hormone (GnRH) neurons are responsible for control of reproduction and reproductive behavior and neuronal size has been directly linked to function. Low dose, chronic BPF exposure (0.25 μM) of developing zebrafish embryos showed a significant decrease in size of GnRH neurons (Weiler & Ramakrishnan, 2019).

Additionally, 0.2 μM ß-estradiol treatment mimicked effects of BPF on GnRH neurons and the combined treatment of BPF (0.25 and 0.5 μM) and estrogen receptor antagonist ICI (1μM) showed that ICI mitigated effects of BPF, implicating the estrogen pathway as a target of BPF. While BPA toxicity has been extensively studied and characterized, this is not the case for its analogues. Together, these studies highlight the potent endocrine disrupting capabilities of BPA analogues particularly through estrogenic and oxidative stress pathways and call into question their safety for widespread usage.

Parabens: Recently, parabens, a class of EDCs commonly used as preservatives in cosmetic products, have been studied as potential breast carcinogens. Methylparaben, the most commonly used of this class of chemicals, is detectable in urine samples in 99.1% of individuals in the United States, as well as human breast tissue and breast milk (Barr, Metaxas, Harbach, Savoy, & Darbre, 2012; Calafat, Ye, Wong, Bishop, & Needham, 2010). At 0.1 mg/kg/day, the estimated dose corresponding to the 95th percentile of human exposure, methylparaben exposure during puberty increases mammary glandular tissue and mammary tissue gene expression of genes related to DNA repair and replication in Sprague-Dawley rats (Gopalakrishnan et al., 2017). In the BT-474 ERα+/HER2+ cell line, 2 hour treatments of butylparaben (1, 10, and 100μM) increased the potency of heregulin regulation of cell proliferation and expression of *cmyc*, a stem cell pluripotency factor (Pan et al., 2015). Overexpression of human epidermal growth factor receptor-2 (HER2), a heregulin receptor, increases the breast cancer stem cell population of both HER2+ and HER2-breast tumors (Korkaya & Wicha, 2013). These studies suggest that exposure to parabens effect breast stem cell differentiation and proliferation, however, further studies specifically measuring alterations in the stem cell population would provide more insight.

In addition to modulating breast development and breast cancer cell proliferation, parabens have been shown to induce adipogenesis in multiple cell lines and in human adipose derived stem cells (Hu et al., 2013, 2017). Methylparaben exposure leads to increased body weight, total white adipose tissue weight, and leptin mRNA expression in C57BL/6J mice, albeit at a high dose (100 mg/kg/day, oral gavaged daily for 12 weeks) (Hu et al., 2016). Leptin, adiponectin, and other adipokines that play a role in inflammation have differential effects on breast stem cell self-renewal (Esper et al., 2015; Hill et al., 2019). Inflammation plays a physiological role in stem cell self-renewal and differentiation during wound healing, as discussed above. Exposure to environmental chemicals that results in increased inflammatory signaling may have downstream effects on stem cell proliferation, differentiation, and turnover.

Perfluoroalklyl substances (PFAS)—PFAS are a class of surfactants used industrially and commercially and are becoming of increasing concern to human health due to their environmental persistence even after being phased out of production. Most commonly used are perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) whose endocrine disrupting capabilities have recently been studied both *in vitro* and *in vivo*. Exposure of T47D hormone-dependent breast cancer cells to PFOS or PFOA (10^{-12} to 10^{-4} M) alone showed no activation of estrogenic activity and no effect on cell proliferation (Sonthithai et al., 2016). Interestingly, co-incubation of T47D cells with PFOS (10^{-10} to 10^{-7} M) or PFOA

($10^{-9}$to 10^{-7} M) and 1 nM of 17β-estradiol (E2), resulted in enhanced E2-induced estrogen response element transcriptional activation, E2-induced proliferation, ERK 1/2 activation, and upregulation of E2 responsive gene *pS2* (Sonthithai et al., 2016). Similarly, human breast epithelial cells (MCF10A) exposed to PFOA (100μM) or PFOS (10μM) showed no change in ERα or ERß protein expression relative to control (Pierozan & Karlsson, 2018). However, PFOA (50 and 100 μM) and PFOS (1 and 10 μM) enhanced cell proliferation through cell cycle acceleration of the G_0/G_1-to-S phase transition and stimulated cell migration and invasion (Pierozan & Karlsson, 2018), demonstrating the potential for these chemicals as inducers of neoplastic transformation. Additionally, 30 minutes pre-incubation of PFOA treated cells with estrogen receptor antagonist ICI 182,780(100nM) showed no effect but pre-incubation with PPARα antagonist GW 6471 (1μM) prevented MCF-10A proliferation, indicating that PFOA likely works through a PPARα mechanism instead of an estrogenic pathway (Pierozan & Karlsson, 2018). Female offspring of CD-1 and C57Bl/6 mice exposed to low dose PFOA (0.1–1mg/kg) during gestational days 1–17 experienced delays in pubertal mammary gland development exhibited by defects in terminal end budding and branching (Tucker et al., 2015). White and colleagues also showed that gestational PFOA exposure of 5 mg PFOA/kg BW/day exposure by oral gavage (GD 1–17, 8–17, or 12–17) alters mammary epithelial differentiation in CD-1 dams in addition to female pups (White et al., 2006). In dams, PFOA exposure resulted in delayed mammary differentiation and epithelial involution as well as alterations in milk protein gene expression. PFAS exposure poses significant risk to human health, particularly during crucial stages in development, and this body of work displays the need to prevent and regulate PFAS exposure.

Pesticides

Dichlorodiphenyltrichloroethane (DDT) and Organochlorine Pesticides: The evidence linking pesticide exposures to cancer at various tissue sites is mounting (Mostafalou & Abdollahi, 2013). Organochlorines (OCs) are environmentally persistent insecticides, many of which were banned worldwide over 20 years ago (Eldakroory et al., 2017). DDT is an environmentally persistent chemical that is still used today in regions with high vector-borne disease burdens to eradicate insects such as mosquitoes (International Agency for Research on Cancer, 2015; Sapbamrer et al., 2008). In a comparative cross-sectional study on breast cancer patients in Egypt breast tissue samples, researcher extracted organochlorine pesticides from the samples using gas chromatography (Eldakroory et al., 2017). In this study, researchers described how OCs like DDT, are still commonly used throughout the world and are widely detected in water, soil, milk, and produce (Eldakroory et al., 2017). More specifically researchers described how hexa-chlorobenzene, Chlordane, Methoxyclor, and DDT are all correlated with an increase in malignancies most likely due to tumor promotion, immunosuppression, and endocrine disruption (Eldakroory et al., 2017). Pesticide sprayers in the AGRICAN study, a study focused on farmworkers in the United States, have increased incidence of prostate cancer (SIR = 1.07, 95%CI 1.03–1.11) and non-Hodgkin lymphoma (SIR = 1.09, 95%CI 1.01–1.18) among males (Alavanja et al., 2013; Lemarchand et al., 2017).

DDT is also an EDC which has been linked to early puberty, reproductive abnormalities, and germline cancers (Strong et al., 2015). DDT is also a known carcinogen in humans and commonly still used in lower/middle income counties (Aamir, Khan, & Li, 2018; Sapbamrer et al., 2008). Exposure to OCs, like DDT, has also been associated with multiple cancers epidemiologically and mechanistically including prostate cancer, breast cancer, and liver cancer (Alavanja, Ross, & Bonner, 2013; Cohn et al., 2015; Eldakroory et al., 2017; Lemarchand et al., 2017; Medjakovic et al., 2014; Rusiecki et al., 2017; VoPham et al., 2017; Wong et al., 2015). In a case-control study nested in a prospective 54-year follow-up study of 9,300 daughter in the Child Health and Development Studies pregnancy cohort, 354 controls were matched by birth year to 118 breast cancer cases under the age of 52 years (Cohn et al., 2015). Overall, this study found that even after controlling for the mothers' lipids, weight, race, age, and breast cancer history, women with the highest quartile of exposure to DDT *in utero* had a 3.7 fold increase in breast cancer relative to women in the lowest quartile (Cohn et al., 2015). Exposing prostate cell lines (LNCaP) to p,p-DDT and p,p-DDE (increasing concentrations from 10nM to 20μM) reduces prostate specific antigen levels, which can potentially cause false negatives in prostate cancer screening (Wong et al., 2015). In Strong et al., MSCs were exposed to DDT (100pM, 1nM, 10nM, 100nM, 1μM, or 10μM) and assessed for changes in self-renewal, proliferation and differentiation potential (Strong et al., 2015). Overall, DDT altered MSC morphology and inhibited self-renewal (Strong et al., 2015). There was also a 2.1 fold increased in osteogenic and a 1.8 fold increase in adpiogenic differentiation in 1μM DDT exposed MSCs, accompanied by increased gene expression of differentiation markers Osteonectin, CBFA-1, c-FOS, LpL PPAR-γ, and Leptin. Co-treatment of MSCs undergoing with the anti-estrogen Fulvestrant and DDT decreased markers of both adipogenesis and osteogenesis, pointing to ER signaling as an important target of DDT on stem cell differentiation (Strong et al., 2015).

In another study, researchers used the Agriculture Health Study to analyze Long Interspersed Nucleotide Element 1 (LINE1) methylation differences among male pesticide applicators (Rusiecki et al., 2017). LINE1 elements are non-coding repeated sequences commonly used to represent global methylation (Rusiecki et al., 2017). In total, methylation associations with 57 pesticides were analyzed, of which 10 pesticides had a positive association with DNA methylation of LINE1 elements (atrazine, dicamba, imazethapyr, terbufos, fenthion, heptachlor, butylate, EPTC, DDVP, and metolachlor), whereas 8 had a negative association (methyl bromide, carbaryl, chlordane, methyl parathion, ethylene dibromide, paraquat, silvex, and 2,4,5-trchlorophenoxyacetic acid) (Rusiecki et al., 2017). Overall, OCs and arsenicals were found to most be related to methylation alterations (Rusiecki et al., 2017). Although the researchers are unsure of mechanism, they suggested endocrine disruption or generation of ROS can reduce methyltransferase activity which can cause a difference in the global DNA methylation (Rusiecki et al., 2017). Overall, DDT and other organochlorine pesticides display an emerging link to numerous cancers potentially due to alterations in stemness and epigenetic reprogramming.

Chlorpyrifos (CPF): Chlorpyrifos is an organophosphate pesticide which has been associated with adverse neurodevelopmental outcomes and stem cell biology in multiple tissues (Rauh et al., 2011). Researchers studying a pesticide mixture of CPF-ethyl,

dimethoate, diazinon, iprodione, imazalile, manb, and mancozeb found these chemicals to be associated with premature aging of MSCs at low doses (Hochane et al. 2017). Through a mitochondrial stress analysis, ROS was found to increase with increasing pesticide mixture doses, and additionally a dose response effect was observed in adipocyte transformations (Hochane et al. 2017). Overall the researchers were unsure if stemness was altered in MSCs considering the cells still expressed SOX2 and OCT4 (Hochane et al. 2017). Exposure of female Sprague-Dawley rats to environmentally relevant doses of chlorpyrifos (from 0.01 to 2.5 mg/kg/day) led to an increase in proliferation and a higher number of ducts in their mammary glands, reflecting altered differentiation potentially linked to an estrogenic effect (Nishi & Hundal, 2013; Clara Ventura et al., 2016). CPF exposure (0.05, 0.5, 5, or 50μM) in the N-nitroso-*N*-metilurea-induced cancer model led to decreased tumor latency and increased tumor incidence, as well as increases in expression of the gene for histone deactylase 1, suggesting that chlorpyrifos may also act through epigenetic mechanisms (C. Ventura et al., 2019). More research is necessary to understand the impact of pesticide exposures, particularly during developmental windows of susceptibility, on stem cell regulation and cancer risk.

BIOLOGICAL EXPOSURES

Human Papillomavirus (HPV)—Based on the US National Health and Nutrition Examination Survey (NHANES) data, oral HPV has a prevalence of 6.9% in men and women from 14 to 69 years of age (95% CI: 5.7–8.3%) (Gillison et al., 2012). HPV is the primary cause of a subset of oropharyngeal squamous cell carcinomas (OSCCs) (Gillison et al., 2012). There are over 160 strains of HPV, and HPV16 and HPV18 are considered the most oncogenic with 50 to 90% of HPV-positive head and neck squamous cell carcinomas (HNSCCs) being due to HPV16 (Gillison et al., 2012; Pullos, Castilho, & Squarize, 2015). Using the ALDH as a biomarker of cancer cell stemness in OSCCs related to HPV16, there was a 62.5 fold greater frequency of CSCs in HPV16-positive OSCCs than HPV negative OSCCs (M. Zhang et al., 2014). When isolating CSCs in culture from HPV-positive and HPV-negative HNSCCs, there was, however no statistically significant difference in CSC proportions (Tang et al., 2013). In general there is still some uncertainty within the field of HPV-positive HNSCCs and CSC occurrence and further research is needed (Pullos et al., 2015).

In HPV-associated cervical cancers, there are mutations that are associated with CSCs and HPV (Pullos et al., 2015; Tyagi et al., 2016). High-risk HPV strains HPV16 and HPV18 code for viral oncoproteins E6 and E7 that interfere with the regulatory proteins p53 and retinoblastoma (Münger et al., 1989; Tyagi et al., 2016; Werness, Levine, & Howley, 1990). The HPV oncoprotein E6 has been found to control stemness and self-renewal through upregulation of HES1 in cervical cancer cells from both primary tissue and cell line (HeLa) xenografts into mice (Tyagi et al., 2016). HES1 is a downstream gene of Notch1 which is responsible for stemness, and through blocking E6 in both primary tissue and cell line xenografts, HES1 became blocked (Tyagi et al., 2016). Other factors associated with stemness of cervical CSCs, like ABCG2 receptor and cervicosphere formation in culture, were also increased in HPV-positive cervical cancers (Tyagi et al., 2016).

Hepatitis—Hepatocellular carcinomas (HCC) are cancers primarily due to Hepatitis B or C infection, but are also associated with liver disease and alcohol abuse (Torre et al., 2015; Xiong et al., 2019). Overall in developing countries 32% of infection-related cancers are due to hepatitis B (HBV) and hepatitis C (HCV), whereas in more developed countries, HBV and HCV accounts for 19% of all infection related cancers (Xiong et al., 2019). In a study aimed at determining HCV and HBV mechanisms for creating aberrant transcriptional enhancers, researchers measured hypomethylation among HCC patients in Hong Kong at the Prince of Wales Hospital (Xiong et al., 2019). When observing the methylome of HCC patients, specific enhancer C/EBPβ hypomethylation was correlated with a shorter survival rate (HR: 4.4, $p < 0.005$) (Xiong et al., 2019). When C/EBPβ was deleted, the global enhancer activity reduced causing less invasion and colony formation (Xiong et al., 2019). Clinicopathological assessment of human liver tumors has also consistently identified an expansion of stem-like oval cells in hepatitis-associated cancers (Hsia, Evarts, Nakatsukasa, Marsden, & Thorgeirsson, 1992). Oval cell activation is an important first step in liver regeneration, as oval cells are bipotent cells that can differentiate into both bile duct epithelial cells and hepatocytes (Erker & Grompe, 2008). A major hypothesized mechanism for this expansion is the increase in inflammatory signaling within the stem cell niche, driven by infiltrating immune cells (Libbrecht, Desmet, Van Damme, & Roskams, 2000)

PHYSICAL HAZARDS

Some of the best evidence supporting the windows of susceptibility hypothesis in human populations comes from studies of the cohort of survivors of the atomic bomb blasts at Hiroshima and Nagasaki. Cancer incidence is elevated for survivors across multiple tumor types including thyroid (Prentice & al, 1982), breast (Land et al., 2003), and skin (Ron et al., 1998). For each of these tumor types, early life atomic bomb radiation exposures were substantially more associated with later life cancer incidence than exposure later in life, which shows that the effects of early life radiation exposure persist throughout the life course (Douple et al., 2011). Multiple forms of radiation have been linked to cancer. Sunlight is a necessary part of human health especially because it is essential for the synthesis of Vitamin D in the body (Notara et al., 2015). More specifically, there are three types of UV light: UVA light is defined as 315–400nm, UVB light is 280–315nm and UVC is 100–280nm (Notara et al., 2015; Wong, Ranganath, & Kasko, 2015). UVB and UVC light are known to cause DNA damage, skin inflammation, ageing of the skin, and melanoma (Notara et al., 2015; Wong et al., 2015). UV light is also commonly used for sanitation in the biotechnology, laboratory science, and medical fields (Wong et al., 2015). There is a large literature on UV light and how it affects human stem cells. In Wong et al. 2015, researchers compared UVB and UVC light since UVA is known to cause indirect DNA damage by producing free radicals (Wong et al., 2015). Researchers compared decoupled UV and radical generation in 2D and 3D cell cultures of human mesenchymal stem cells (Wong et al., 2015). Overall, radical polymerization had a large variable effect on gene expression, primarily in the 3D samples. p53 was also affected in UV waves shorter than 254nm by effecting DNA repair. p53 also directly regulates EMT plasticity (Wong et al., 2015). *TP53* was found to be upregulated in the UV condition in comparison to no UV ($p=3.72 \times 10^{-25}$) (Wong et al., 2015). Although p53 itself was not statistically significant between exposed and unexposed 3D cultures, the researchers still determined UVA exposure does not cause

differentiation but this suggests it could lead to cancer by encouraging cancer stem cell growth (Wong et al., 2015). In another study, researchers found that UVB light induced the Wnt7a and Notch pathways in human skin (Fukunaga-Kalabis et al., 2015). Wnt7a upregulates Notch inhibitor NUMB in neural crest stem-like cells (Fukunaga-Kalabis et al., 2015). Wnt and Notch signaling played an essential role in self-renewal and differentiation of neural crest stem cells and inhibition of Notch can impair proliferation and cause cell death. NUMB depletion also increased the expression of Notch target genes *HES1*, *HEY1*, and *WNT5a* (Fukunaga-Kalabis et al., 2015). Overall, Wnt7a was upregulated by UVB irradiation in keratinocytes although base levels were achieved within 3 days. UV light experiments have also been tested in the limbal epithelial stem cells, present at the junction of the cornea and sclera in the human eye (Notara et al., 2015). Notara et al. 2015 found that UVB light induced apoptosis in the limbal epithelial stem cells and reduced the metabolic activity of both the epithelial cells and the limbal fibroblasts (Notara et al., 2015). UVB also altered the pro-angiogenic and pro-inflammatory profile by altering angiogenin, IGFBP-3, VEGFC, VEGFA, MCP1, TNF_{α}, and IFN_{γ}, highlighting the link between alterations in inflammatory cytokines and expression of stem cell potency factors (Notara et al., 2015)

3. Future Directions

We have reviewed the impact of a range of chemical, biological, and physical hazards on pathways and processes relevant to stem cells and cancer. While the evidence linking these exposures and cancer risk and dysregulated stemness is mounting, these exposures only represent the tip of the iceberg of the relevant external environment. There are over 80,000 chemicals in commerce that are registered with the EPA, however, our assessment of the carcinogenicity of these chemicals has been very limited (Schmidt, 2016). Further, we know that poor diet quality and obesity can modify many of the same stem cell regulating mechanisms as the environmental factors profiled above (Lengyel, Makowski, DiGiovanni, & Kolonin, 2018). Emerging research is also pointing to the social environment as a significant predictor of cancer risk. Socioeconomic status is related to chronic stress and poor cancer outcomes, likely due to exposure to crowding, crime, noise pollution, discrimination, and lack of access to healthcare (Baum, Garofalo, & Yali, 1999; Dreyer, Nattinger, McGinley, & Pezzin, 2016; Sallis, Floyd, Rodriguez, & Saelens, 2017). These chronic exposures have been associated with an increased allostatic load, reflecting elevated endocrine and inflammatory responses (Graves & Nowakowski, 2017). People who were raised in low socioeconomic status areas have higher allostatic loads in adulthood, suggesting that the social environment may program health outcomes throughout the life course (Graves & Nowakowski, 2017). Additionally, people residing in low income populations have less physical activity each day due to fewer bicycling and walking areas and heavy traffic (Sallis et al., 2017). Individuals of lower socioeconomic status are also more likely be exposed to high levels of air pollution (Hajat, Hsia, & O'Neill, 2015), which has been classified as a known human carcinogen by the International Agency for Research on Cancer (Loomis et al., 2013). These environmental disparities have profound impacts on population health, and alterations of stem cell regulation may provide an intersection point for molecular impacts relevant to cancer risk (Figure 3).

There are a number of persistent cancer disparities where we do not fully understand the biological underpinnings. For example, women of African ancestry are two to three times more likely to be diagnosed with the most aggressive subtype of breast cancer, triple negative breast cancer, compared to women of European ancestry (Carey et al., 2006). These disparities are hypothesized to be driven by complex interactions between genetic susceptibility and the chemical, dietary, and social environment (Dietze, Sistrunk, Miranda-Carboni, O'Regan, & Seewaldt, 2015). Studies of normal breast tissue have identified that women of African ancestry have enrichment of CD44+/CD24-stem cells, relative to tissues from women of European ancestry (Nakshatri et al., 2015). Whether these differences in the underlying stemness in the normal tissue are linked to genetic factors, environmental exposures, or an interaction between these remains an important unanswered research question with important implications on our understanding of risk. To establish the link between the environment, stem cells, and cancer, we envision advances in three broad areas to be essential: (1) Assessment of the molecular effects of an expanded set of environmental exposures on stem cells and their microenvironments using tools such as patient derived organoid cultures, genetically modified animal models, and single cell analyses, (2) Optimization of new tools in molecular epidemiology to assess stem cell specific alterations in well-characterized population-based cohort studies, allowing researchers to pose complex questions about exposure interactions at specific life stages and cancer risk, and (3) Development of new interventions to target the stemness state in both cancer prevention and treatment. Defining the environmental impact on stemness throughout the life course and developing new agents for targeting stem cells will provide us with significant opportunities to reduce cancer disparities and improve public health.

Acknowledgements

This work was supported by grants from the National Institute of Environmental Health Sciences (R01ES028802, T32ES007062, P30ES017885), the National Cancer Institute (P30CA046592), the Educational Research Center by the National Institute of Occupational Safety and Health (T42OH008455), the University of Michigan Forbes Institute for Cancer Discovery, the University of Michigan Institute for Data Science, the Ravitz Family Foundation, and a Merit Fellowship from the University of Michigan Rackham Graduate School (to CAF).

Abbreviations

ALDH
Aldehyde dehydrogenase

BPA, BPB, BPF, BPAF, BPS
Bisphenol-A, -B, -F, -AF, -S

CBC
crypt base columnar cell

CPF
chlorpyrifos

CSC
cancer stem cell

DDT
Dichlorodiphenyltrichloroethane

DES
diethylstilbestrol

EDC
endocrine disrupting chemical

EMT
epithelial-to-mesenchymal transition

ER
estrogen receptor

GnRH
gonadotropin releasing hormone

HBV/HCV
hepatitis B/C virus

HCC
hepatocellular carcinoma

HER2
human epithelial growth factor receptor-2

hfSCs
hair-follicle stem cells

HNSCC
head and neck squamous cell carcinoma

HPV
human papilloma virus

iPSCs
induced-pluripotent stem cells

Lgr5
Leucine-rich repeat containing G-protein coupled receptor-5

LINE
long interspersed nucleotide element

MET
mesenchymal-to-epithelial transition

MSCs
mesenchymal stem cells

OC
organochlorine

OSCCs
oropharyngeal squamous cell carcinomas

PFAS
perfluoroalkyl substances

PFOA
perfluorooctanoic acid

PFOS
perfluorooctane sulfonate

ROS
reactive oxygen species

VIM
vimentin

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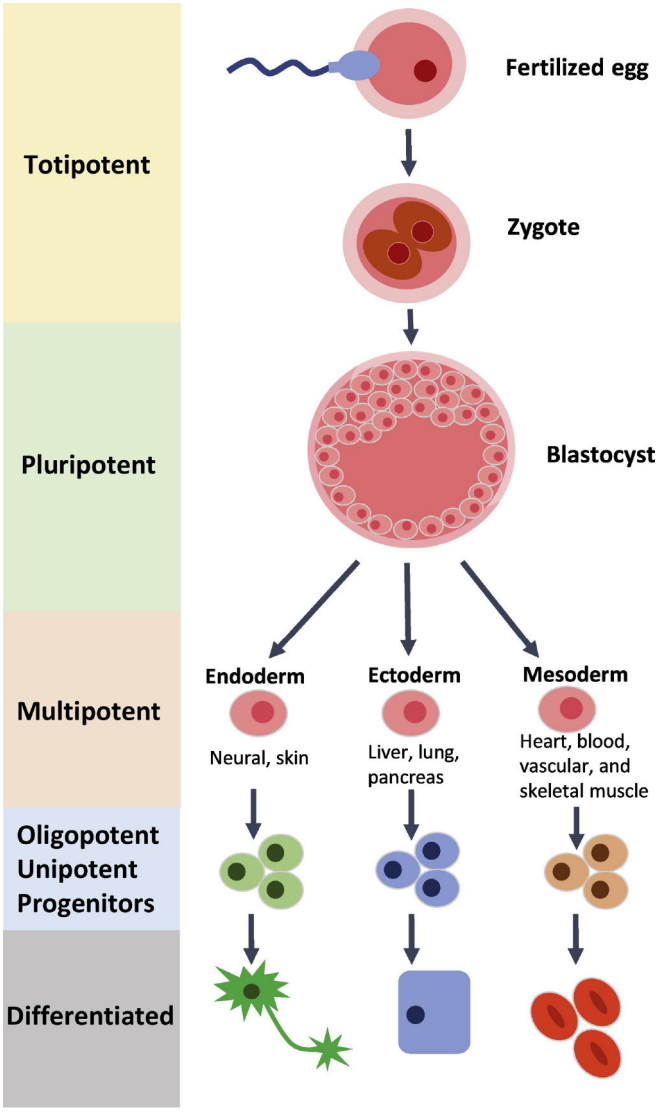

Figure 1. Stem cell hierarchy in embryonic differentiation.

The trajectory of a single fertilized egg differentiating into tissue specific lineages. Stem cell potency decreases as differentiation occurs and cells progress down the hierarchy.

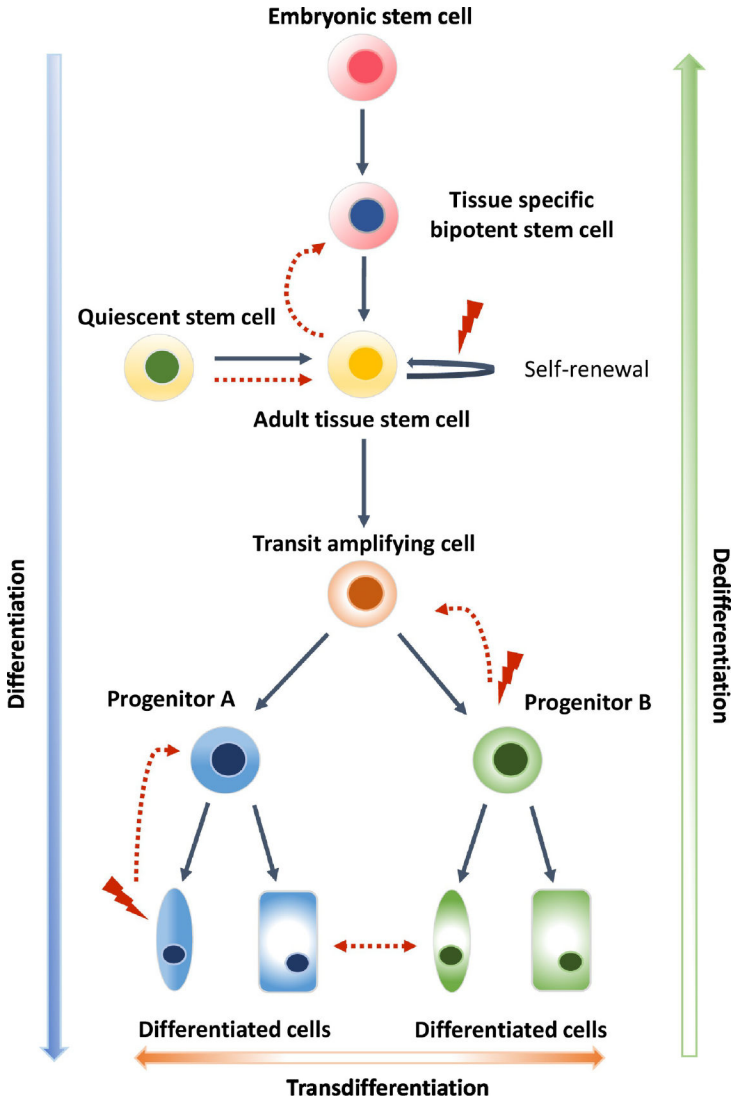

Figure 2. Multi-directional differentiation of plastic stem cells in response to environmental conditions.

In addition to differentiation down the normal hierarchy, stem cells are able to de-differentiate upwards into less differentiated precursors. Differentiated cells, such as in the intestines, are able to trans-differentiate into differentiated cells of another lineage. Gray arrows depict traditional hierarchical differentiation, dashed red arrows indicate deviations from traditional differentiation, lightning bolts indicate processes potentially impacted by environmental stressors.

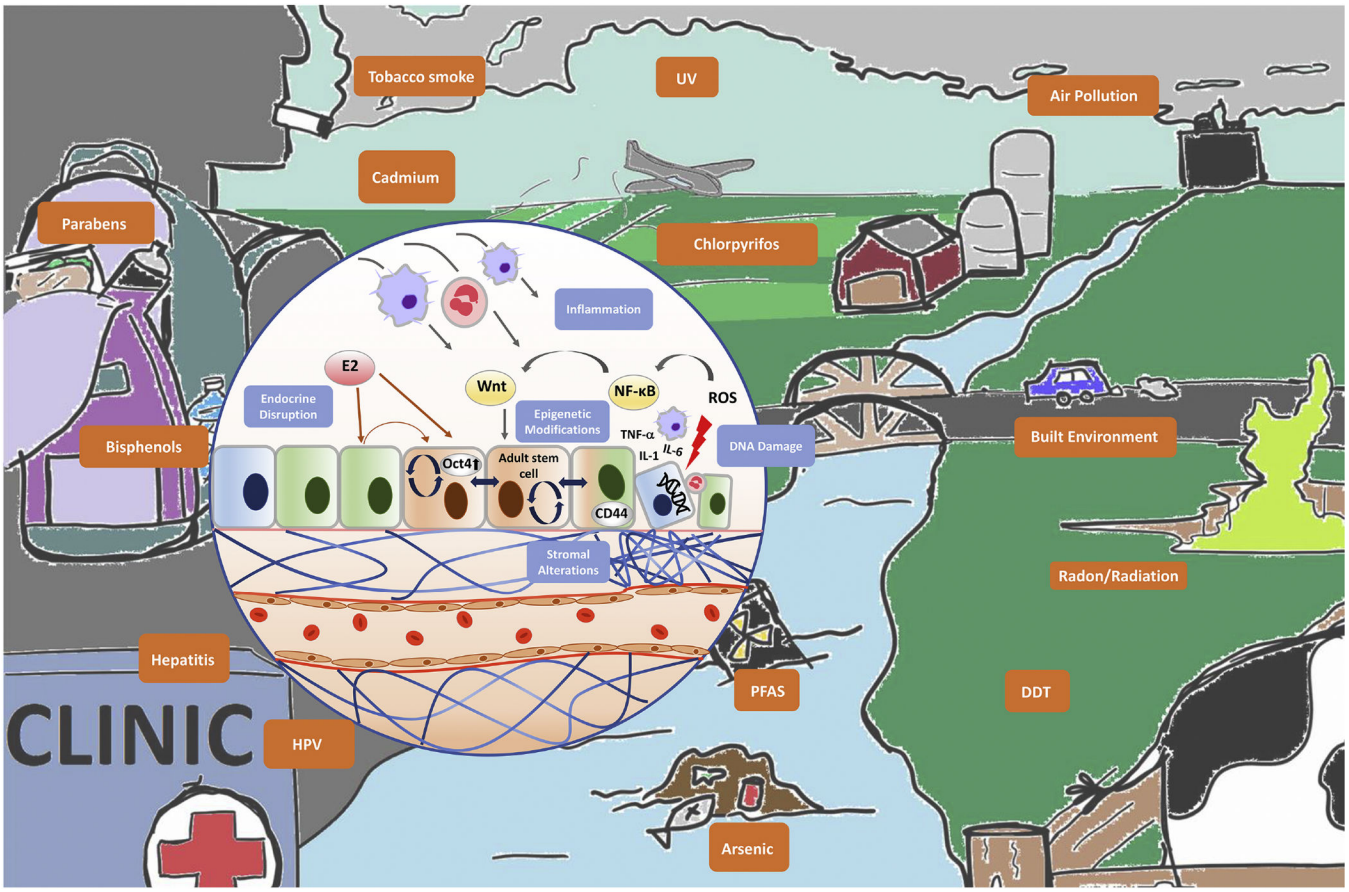

Figure 3. Potential environmental exposures that may affect the stem cell microenvironment.

The background image encapsulates the biological, physical, and chemical exposures found in the environment. Exposures are presented in the image and labeled with orange text boxes. The zoomed in circle presents the stem cell microenvironment and the pathways of environmental dysregulation of stem cell biology are presented in blue text boxes.