Actions of Estrogens and Endocrine Disrupting Chemicals on Human Prostate Stem/Progenitor Cells and Prostate Cancer Risk

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Abstract

Estrogen reprogramming of the prostate gland as a function of developmental exposures (aka developmental estrogenization) results in permanent alterations in structure and gene expression that leads to an increased incidence of prostatic lesions with aging. Endocrine disrupting chemicals (EDCs) with estrogenic activity have been similarly linked to an increased prostate cancer risk. Since it has been suggested that stem cells and cancer stem cells are potential targets of cancer initiation and disease management, it is highly possible that estrogens and EDCs influence the development and progression of prostate cancer through reprogramming and transforming the prostate stem and early stage progenitor cells. In this article, we review recent literature highlighting the effects of estrogens and EDCs on prostate cancer risk and discuss recent advances in prostate stem/progenitor cell research. Our laboratory has recently developed a novel prostasphere model using normal human prostate stem/progenitor cells and established that these cells express estrogen receptors (ERs) and are direct targets of estrogen action. Further, using a chimeric *in vivo* prostate model derived from these normal human prostate progenitor cells, we demonstrated for the first time that estrogens initiate and promote prostatic carcinogenesis in an androgen-supported environment. We herein discuss these findings and highlight new evidence using our *in vitro* human prostasphere assay for perturbations in human prostate stem cell self-renewal and differentiation by natural steroids as well as EDCs. These findings support the hypothesis that tissue stem cells may be direct EDC targets which may underlie life-long reprogramming as a consequence of developmental and/or transient adult exposures.

Highlights

- Early-life estrogens and EDC exposures heighten susceptibility for prostate carcinogenesis with aging.
- Human prostate epithelial stem and early stage progenitor cells express ERs and are direct targets for estrogenic actions.
- Estrogens, retinoids and EDCs modulate human prostate stem cell self-renewal and differentiation capabilities.
- This is the first evidence to demonstrate that prostate stem/progenitor cells are EDC targets which may underlie life-long increased carcinogenic risk.

Key words

Estrogen, endocrine disrupting chemicals, endocrine disruptors, prostate stem cells, prostate progenitor cells, prostate cancer

Abbreviations

E2: estradiol; EDCs: endocrine disrupting chemicals; AR: androgen receptor; ER: estrogen receptor; PIN: prostate intraepithelial neoplasia; PCBs: Polychlorinated biphenyls; PCDDs: polychlorinated dibenzo-p-dioxins; POPs: persistent organic pollutants; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR: aryl hydrocarbon receptor; PSA: prostate specific antigen; PSCA: prostate stem cell antigen; ABCG2: a member of the ATP binding cassette (ABC) transporters; BCRP: breast cancer resistance protein; HSC: hematopoietic stem cells; DHT: dihydrotestosterone; HGF: hepatocyte growth factor; PR: progesterone receptor.

Acknowledgements

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer deaths in North American men (Jemal et al., 2008). It is known that steroids play a role in the initiation and progression of prostate cancer, which is the basis for hormonal treatment strategies that include androgen ablation and androgen receptor (AR) blockade (Eisenberger et al., 1998, Huggins and Hodges, 1941). Increasing evidence indicates that in addition to androgens, estrogens play key roles in prostate carcinogenesis and progression, although the mechanisms are not fully understood (Ellem and Risbridger, 2007, Hu et al., 2011, Leung et al., 2010, Nelles et al., 2011, Prins et al., 2007, Prins and Korach, 2008). In men, chronically elevated estrogens have been associated with increased risk of prostate cancer (Modugno et al., 2001) while in rodents, estrogens in combination with androgens induce prostate cancer (Bosland, 1996). It is recognized that age, race, genetics (family history), diet, and environmental factors can impact prostate cancer risk (Reuben et al., 2010). Endocrine disrupting chemicals (EDCs) are a class of environmental toxicants that interfere with endocrine signaling pathways. In addition to direct effects in adults, strong evidence indicates that developing tissues are particularly sensitive to EDCs and that early-life EDC exposures promote specific disorders in adults (Foran et al., 2002, Heindel, 2005), a phenomenon referred to as the developmental basis of adult disease.

Recent advances in stem cell research indicate that stem cells and early stage progenitor cells may be direct carcinogenic targets and the cells of origin in cancer initiation and progression. Together with our previous findings in animal models which show that early-life exposures to natural and environmental estrogens increase susceptibility to prostate carcinogenesis through structural and epigenomic reorganization (Ho et al., 2006, Prins, 1992, Prins and Birch, 1994, Prins et al., 1996, Prins and Ho, 2010, Prins et al., 2008, Prins et al., 1993, Prins et al., 2011), we hypothesize that developmental reprogramming of the prostate gland by EDCs may involve epigenomic alterations in prostate stem/progenitor cells during early gland formation, thus predisposing to prostate cancer upon aging. At present, there is a critical need to determine whether early life estrogenic reprogramming of prostate cells similarly occurs in humans. To meet this current need, we have recently developed novel *in vitro* and *in vivo* models using stem and early stage progenitor cells isolated from normal human prostates and used these to initiate hormonal carcinogenesis (Hu et al., 2011). Importantly, these *in vitro* prostasphere and *in vivo* chimeric prostate models with carcinogenic induction can serve as suitable models for examining stem cell perturbations and carcinogenic actions of EDCs on human prostate cells. In the current review, we will briefly assess available evidence for EDCs

and increased prostate cancer risks, discuss recent advances in prostate stem cell research, and present evidence for reprogramming of human prostate stem/progenitor cells by estrogens and EDCs using our novel human prostasphere and chimeric prostate models.

Endocrine Disruptors and Prostate Cancer Risk

In the human population, direct connections between EDCs and prostate cancer are primarily limited to epidemiology studies and *in vitro* analysis using cancer cell lines (Prins, 2008). These findings are supported by *in vivo* studies in animal models that suggest associations between EDCs and prostate cancer, carcinogenesis and/or susceptibility. Herein we will highlight the evidence on EDCs with estrogenic actions. For the sake of simplicity, we here refer to environmental estrogens as molecules with identified estrogenic activity, mostly through activation of ERs or altered estrogen metabolism.

The most compelling data in humans to link prostate cancer with environmental chemicals comes from the established occupational hazard of farming and increased prostate cancer rates which is believed to be a function of chronic or intermittent pesticide exposures (Alavanja et al., 2003, Meyer et al., 2007, Morrison et al., 1993, Van Maele-Fabry et al., 2006). This is supported by a large epidemiology study (Agricultural Health Study) in a collaborative effort between the NCI, NIEHS and EPA (www.aghealth.org) that evaluated >55,000 pesticide applicators in North Carolina and Iowa since 1993 and revealed a direct link between methyl bromide exposure, a fungicide with unknown mode of action, and increased prostate cancer rates (Alavanja et al., 2003). Further, six pesticides (chlorpyrifos, fonofos, coumaphos, phorate, permethrin and butylate) out of 45 common agricultural pesticides showed correlation with exposure and increased prostate cancer in men with a familial history, suggesting gene-environment interactions (Alavanja et al., 2003, Mahajan et al., 2006). Significantly, chlorpyrifos, fonofos, coumaphos, phorate, permethrin are thiophosphates with acetylcholine esterase inhibitor action as well as significant capacity as p450 enzyme inhibitors. In particular, chlorpyrifos, fonofos and phorate strongly inhibit CYP1A2 and CYP3A4 which are the major p450s that metabolize estradiol (E2), estrone and testosterone in the liver (Usmani et al., 2006, Usmani et al., 2003). This raises the possibility that exposure to these compounds may interfere with steroid hormone metabolism and disturb hormonal balance which in turn contributes to increased prostate cancer risk. A similar mechanism of endocrine disruption *in vivo* has been identified for polychlorinated biphenyls (PCBs) and polyhalogenated aromatic hydrocarbons (including dioxins, BPA and dibenzofurans) through potent inhibition of estrogen sulfotransferase which effectively elevates bioavailable estrogens in target organs (Kester et al., 2000, Kester et al., 2002).

Bisphenol A (BPA) is a high volume synthetic monomer used in the production of polycarbonate plastics, epoxy linings of food and beverage cans, and in numerous common household and consumer products. Significant levels of BPA have been found in the urine of 93% of US individuals (Calafat et al., 2008) with highest levels found in infants and children (Calafat et al., 2009, Eddington and Ritter, 2009, Kuroda et al., 2003, Lee et al., 2008). BPA was initially synthesized in the 1890s, however, its estrogenic actions of BPA were identified in 1936 (Dodds and Lawson, 1936). Although its relative binding affinity and activation of nuclear ERα and ERβ are ~1,000 to 10,000 fold lower than E2 or diethylstilbestrol (Kuiper et al., 1998b, Lemmen et al., 2004), BPA activates membrane ERs through non-genomic signaling pathways with an EC$_{50}$ equivalent to E2 (Song et al., 2002, Walsh et al., 2005). Effects of BPA with regards to carcinogenic potential, including the prostate gland, have been reviewed by an expert panel (Keri et al., 2007). In short, there is evidence from rodent models and human prostate cell lines that BPA can influence carcinogenesis, modulate prostate cancer cell proliferation and for some tumors with AR mutations, stimulate progression. Using rodent models, our laboratory has shown that transient, early-life exposure to low-doses of BPA increased susceptibility to adult-onset precancerous lesions and hormonal carcinogenesis. Specifically, neonatal Sprague–Dawley rats exposure to 10 μg BPA/kg BW on post-natal days 1, 3 and 5 significantly increased the incidence and score of adult estrogen-induced prostate intraepithelial neoplasia (PIN), the precursor lesion for prostate cancer, as to compared to control rats (Ho et al., 2006, Prins et al., 2008, Prins et al., 2011). This model of sensitivity to hormonal carcinogenesis is relevant to humans in that relative E2 levels increase in the aging male and may contribute to prostate disease risk (Kaufman and Vermeulen, 2005). Furthermore, these studies identified alterations in DNA methylation patterns in multiple cell signaling genes in BPA-exposed prostates which suggest that environmentally relevant doses of BPA reprogram the developing prostate through epigenetic alterations (Ho et al., 2006, Prins et al., 2008).

PCBs are a class of synthetic, lipophilic, and persistent compounds widely used in the mid-20th century. Although now banned, the general population continues to be exposed to PCBs due to persistence, ubiquity in the environment, and bioaccumulation up the food chain. Measurable levels of serum PCBs are found in the majority of the general population (Patterson et al., 2009). Many PCBs have estrogenic or anti-androgenic activity and may perturb male reproductive activity. An analysis of adipose tissue concentrations of PCBs in Swedish men with and without prostate cancer revealed a significant association between PCB

levels in the higher quandrants and prostate cancer odds ratio with the most marked associations for PCB153 and trans-chlordane (Hardell et al., 2006). An epidemiologic study of capacitor manufacturing plant workers highly exposed to PCBs revealed a strong exposure–response relationship for prostate cancer mortality (Prince et al., 2006). This supports previous findings of correlations between PCB 153 and 180 and prostate cancer risk in electric utility workers (Charles et al., 2003, Ritchie et al., 2003). While these studies suggested an association between PCB exposure and prostate cancer, no association was reported between PCBs and prostate cancer in a recent Canadian study (Aronson et al.). Further investigation is thus warranted for PCBs and prostate cancer risk.

Cadmium is classified as a human carcinogen by the International Agency for Research on Cancer and the National Toxicology Program. While some large epidemiologic reports have indicated a relationship between cadmium exposure and prostate cancer rates, others have refuted these findings (Parent and Siemiatycki, 2001, Waalkes, 2000). The basic metal cationic portion of cadmium is responsible for both toxic and carcinogenic activity, and the mechanism of carcinogenicity appears to be multifactorial (Huff et al., 2007). Cadmium is known to ligand to ERs and function as an estrogenic mimic. Cadmium has proliferative action with human prostate cells *in vitro* through an ER-dependent mechanism (Benbrahim-Tallaa et al., 2007a). Since cadmium bioaccumulates, further epidemiologic analysis of cadmium and prostate cancer risk is warranted, particularly in men with occupational exposures.

Inorganic arsenic is a metalloid ubiquitously distributed in nature. Environmental inorganic arsenic was first associated with prostate cancer in Taiwanese men in the late 1980s (Chen et al., 1988) and several subsequent studies revealed an association between inorganic arsenic exposure and prostate cancer mortality or incidence (Benbrahim-Tallaa and Waalkes, 2008, Lewis et al., 1999). Importantly, it has been documented that arsenic may mediate some of these effects through endocrine disruption, specifically through interaction with ERs and activation of estrogen-regulated genes (Davey et al., 2007). In this context, there is a recent report that arsenic can induce malignant transformation of prostate epithelial cells *in vitro* and drive them toward an androgen-independent state (Benbrahim-Tallaa et al., 2007b). Since these actions were mediated through Ras-MAPK pathways, it is likely that membrane ERs are involved.

Dioxins [polychlorinated dibenzo-*p*-dioxins (PCDDs)], resist degradation and are thus considered persistent organic pollutants (POPs). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic dioxin and causes a variety of effects, including immunotoxicity, hepatotoxicity, teratogenicity, and tumor promotion (Kuroda et al., 2005). Changes in gene expression induced

by TCDD and related chemicals are initiated by binding to the aryl hydrocarbon receptor (AhR) (Kuroda et al., 2005) and crosstalk between AhR and ERα is well established. Activated AhR inhibits ER activity through several mechanisms, whereas ERα has a positive role in AhR signaling (Matthews and Gustafsson, 2006). Additionally, an inverse relation was found between serum TCDD levels and serum testosterone in chemical production workers (Egeland et al., 1994). Adult TCDD exposure at general population levels is associated with a decreasing risk of BPH with higher exposure levels (Gupta et al., 2006a, Gupta et al., 2006b). Further, TCDD increased tumor-free survival in transgenic TRAMP mice that spontaneously develop prostate cancer while AhR activation decreased lymph node metastasis suggesting that TCDDs may protect against prostate cancer in adulthood (Vezina et al., 2009). In contrast, *in utero* exposure to TCDD in mice interferes with prostate bud patterning and is associated with hyperplastic lesions in aged animals. Together, these findings suggest that timing of TCDD exposures may dictate their effects on subsequent prostate disease (Vezina et al., 2009).

Prostate stem/progenitor cells

Primary prostate epithelial cells include small number of stem/progenitor cells

The prostate gland contains a simple columnar epithelium with three differentiated cell types - basal, luminal, and neuroendocrine cells - that are embedded in a fibro-muscular stroma (Isaacs et al., 1981, Wang et al., 2001). The major epithelial cell population is luminal secretory cells which express cytokeratins (CK) 8/18 and AR and are androgen-dependent for growth, survival and production of secretory proteins such as prostate specific antigen (PSA). Basal epithelial cells are interspersed along the basement membrane and express p63 and CK5 but are largely AR negative and androgen-independent. The scarce population of neuroendocrine cells are scattered within the basal layer and are identified by the expression of chromogranin A and synaptophysin. Strong evidence now exists for the presence of a rare population of adult stem cells within the human prostate gland that are capable of self-renewal as well as differentiation into early progenitor cells that give rise to the 3 differentiated epithelial cell populations (Burger et al., 2005, De Marzo et al., 1998, Leong et al., 2008, Miki and Rhim, 2008, Wang et al., 2009). Rare intermediate cells positive for combinations of CK5 and CK8/18 and prostate stem cell antigen (PSCA) are thought to represent the progenitor or transit amplifying cells (Garraway et al., 2003, van Leenders et al., 2000, Wang et al., 2001).

It is widely accepted that adult stem cells are involved in normal tissue replenishment throughout life while cancer stem cells support cancer growth (Presnell et al., 2002, Smith et al., 2007). Although the cell(s) of origin for prostate cancer may include luminal, basal,

neuroendocrine, progenitor and stem cells (Kasper, 2008, Wang et al., 2009), it is increasingly evident that the resultant prostate cancers contain cancer stem cells that continuously seed and maintain tumor growth (Gu et al., 2007, Kasper, 2008, Kasper, 2009). Even though conventional therapies for prostate cancer eradicate the majority of cells within a tumor, most patients with advanced cancer eventually progress to androgen-independent, metastatic disease that remains essentially incurable by current treatment strategies. Recent evidence has shown that cancer stem cells are a subset of tumor cells that appear to be therapy-resistant and are responsible for maintaining cancer growth which may be the underlying cause of disease relapse (Lawson and Witte, 2007, Maitland and Collins, 2008). Thus understanding the regulation of both normal stem cells and cancer stem cells may provide new insight into the origin and treatment of prostate cancer.

Primary prostate epithelial cell culture is an essential and initial step in isolating prostate stem/progenitor cells from normal and diseased human prostate tissues (Figure 1A). Fresh prostate tissue from normal organ donors and patients undergoing prostatectomy for prostate cancer or other diseases can be digested, dispersed into single cells and established as primary epithelial cell cultures according to methods characterized by Pheel and others (Peehl, 2003). Immunocytochemical staining using vimentin and cytokeratin markers are typically used for characterization of the established epithelial cultures. Under prescribed conditions, primary prostate epithelial cell cultures can be passaged 3-4 times before they loose their survival and growth potential. With advances in stem cell isolation, new approaches are available to isolate stem cell populations with enhanced self-renewal capabilities from primary prostate epithelial cell cultures that contain mixed cell populations. Of these, flow cytometry and 3-D prostasphere culture are the primary techniques employed (Lukacs et al., 2010a, Xin et al., 2007). Flow cytometry is widely used for cell sorting by labeling cells with surface CD markers. Many molecules have been identified as prostate stem cell markers including Sca-1, CD133, CD44, CD117, CD49f and trop2 (Collins et al., 2001, Goldstein et al., 2008, Leong et al., 2008, Richardson et al., 2003, Vander Griend et al., 2008, Xin et al., 2005), and the number continues to grow. Typically, combinations of multiple stem cell markers are used to isolate prostate stem/progenitor cells by cell sorting. Of note, the markers used to isolate prostate stem/progenitor cells are not fully conserved between human and rodent. In the human prostate, stem/progenitor cells have been enriched based on the expression of integrin $\alpha2/\beta1$, CD44, or CD133. Murine prostate stem cells have been isolated by expression of stem cell antigen-1 (Sca-1), integrin $\alpha6$/CD49f, as well as CD44, CD133. Leong *et al*. first identified

CD117 as a prostate stem cell marker in mice and human and a single murine prostate stem cell defined by the phenotype Lin$^-$Sca-1$^+$CD133$^+$CD44$^+$CD117$^+$ generated a prostate after mixing with urogenital sinus mesenchyme (UGM) and transplantation *in vivo* (Leong et al., 2008). Goldstein *et al*. reported that Trop2 enriched for sphere-forming cells from the mouse and human prostate *in vitro* and that Lin$^-$Sca-1$^+$CD49f^{hi}Trop2^{hi} mouse prostate cells gave rise to basal, luminal and neuroendocrine cells *in vivo* (Goldstein et al., 2008). While widely used for the prostate stem cell research, there are several disadvantages of cell sorting including a relative low cell yield, need of multiple stem cell markers, and cell damage following labeling and sorting.

In terms of functional analysis, prostate stem/progenitor cells can also be assessed using FACS side population analysis. The stem cell side population was first identified in hematopoietic stem cells (HSC) that were enriched amongst heterogeneous cell populations based upon their unique ability to actively efflux Hoechst 33342 (Brown et al., 2007, Goodell et al., 1996). ABCG2 is a member of the ATP binding cassette (ABC) transporters, also know as BCRP (breast cancer resistance protein), which can pump a wide variety of endogenous and exogenous compounds out of cells including Hoechst 33342. Widely expressed in a variety of stem cells, ABCG2 is found to be a molecular determinant of the side population phenotype and is recognized as a universal marker of stem cells (Ding et al., 2010, Zhou et al., 2001). The Hoechst exclusion-based side population assay has proven to be a valuable technique for identifying and sorting stem and early stage progenitor cells in a variety of tissues and species. Importantly, prostate stem/progenitor cells and prostate cancer stem cells are defined by expression of ABCG2, consequently, the side-population assay can be used for the isolation and characterization of putative prostatic stem/progenitor cells from heterogeneous cell populations as shown for 2-D prostate epithelial cell cultures in Figure 2.

A separate approach for isolating stem cells from mixed epithelial cell cultures utilizes a three dimensional (3-D) cell culture system wherein only stem-like cells are capable of survival and proliferation, forming spheroid structures of stem and early-stage progenitor cells. First used to isolate neural stem cells, this model system has now been expanded for the culture of other adult stem cells including the prostate with resultant spheroids referred to as prostaspheres (Hu et al., 2011, Hudson, 2003, Lang et al., 2001, Lukacs et al., 2010b, Xin et al., 2007). Using a matrigel-slurry culture system in our laboratory, only ~0.2%-1% of 2-D cultured primary epithelial cells from normal prostate tissues form free-floating prostaspheres that are clonal in origin (Hu et al., 2011). Direct comparison with stem cells sorted by flow cytometry has shown that only prostate stem cells that express Trop2, CD44, and CD49f

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markers exhibit sphere-forming capacity in a 3D culture system (Garraway et al., 2010). The major advantages of the prostasphere assay are the functional isolation of prostate stem/progenitor cells and the expansion capability of the stem/progenitor cells number *in vitro* which provides multiple research opportunities including analysis of growth and differentiation regulation. For example, Bisson, *et al* has shown that Wnt/β-catenin activation increases prostasphere size and the self-renewal capacity of prostate cancer cells with stem cell characteristics (Bisson and Prowse, 2009). Many key variables contribute to the number and cellular composition of the prostaspheres that form in culture including the age of donor, cell density, culturing techniques and passage number of parental cell lines. At early stages of formation, the prostaspheres consist of committed epithelial stem cells that are actively proliferating but have not yet differentiated into cell lineages. Prostaspheres ~30 μm in diameter and consisting of 20 to 40 cells are visible at day 4 of culture (Figure 1B) and through continuous proliferation, continue to grow with diameters reaching ~80 μm at day 7 (Figure 1C). Labeling of prostaspheres at day 7 using a number of stem cell markers including CD117, CD49f, Trop2 and ABCG2 confirms their stem cell – early progenitor cell status at this early stage (Figure 3). With continued culture through day 10, cells located in the spheroid center begin to differentiate into a luminal cell phenotype (CK8/18+) with basal-type cells (p63+) in the periphery, forming double-layered prostaspheres 100~150 μm in diameter (Figure 1D).

Self-renewal ability and differentiation capacity are two properties of stem cells. Since dysregulation of stem cell differentiation could be the early event of cell transformation and carcinogenesis, a prostate stem cell differentiation assay would be a useful model for studying the carcinogenic potential of EDCs. The features of prostate stem/progenitor cells include quiescence, high proliferative potential and the ability of single cells to give rise to ductal structures that contain both basal and luminal cells. The regeneration ability of normal human prostasphere cells was evidenced by the formation of chimeric prostatic gland tissue *in vivo* following tissue recombination with inductive rat UGM and grafting under the renal capsule of nude mice (Hu et al., 2011). The differentiation of prostate stem/progenitor cells can be achieved in the *in vitro* prostashpere assay system using several different conditions that includes extension of culture for up to 30 days, co-culture with stromal cells and treatment with differentiating factors such as 10 nM dihydrotestosterone (DHT) or 25 ng/ml hepatocyte growth factor (HGF). Using these approaches, our laboratory has been able to drive prostaspheres towards a differentiated phenotype as characterized by CK8/18 and Nkx3.1 labeling of inner cells, detection of AR and PSA gene expression by RT-PCR, CK5 and p63 labeling of

peripheral cells, and formation of ductal branches as well as lumen-like structures, thus recapitulating events in early prostate growth and differentiation (Hu et al., 2011).

Most recently, we have also found that retinoic acid can directly drive prostate stem/progenitor cells into differentiation pathways. Retinoids and retinoic acids are derivatives of vitamin A and are potent regulators of cell proliferation and differentiation through the activation of retinoic acid receptors (RARs) (Dolle et al., 1990, Metallo et al., 2008). Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells through activation of HNF-3α (Jacob et al., 1994). In addition to ERs, we have found that human prostate stem/progenitor cells express high levels of RARs and RXRs and thus are potential retinoid targets. In our human prostasphere culture system derived from normal human prostate epithelial cells, all-trans retinoic acid (100 nM) markedly augmented the differentiation of prostate stem/progenitor cells towards the luminal epithelial phenotype as indicated by the formation of double layered prostasphere structure as early as days 6-7 (Figure 4). Further, retinoic acid strongly upregulated expression of CK 18 and HOXB13, which are involved in luminal epithelial cells differentiation, while repressing p63 expression in the inner spheroid cells (Figure 4). These findings support the multiple studies on the usefulness of retinoids in chemoprevention and treatment for prostate cancer (Huss et al., 2004, Schenk et al., 2009) and suggest that their actions may, in part, be mediated through direct actions on prostate stem cells, driving differentiation and limiting self-renewal. Thus we predict that chemicals which either augment or interfere with retinoid signaling will have the capacity to directly alter human prostate stem cell differentiation capacity with potentially beneficial or detrimental outcomes with regards to prostate health.

Estrogens and EDCs action on human prostate stem/progenitor cells

As the property of self-renewal allows for a long life span of stem cells, undifferentiated stem/progenitor cells are highly susceptible to environmental injuries over time and have the capacity to transmit their "injury memory" to the differentiated progeny (Cheng et al., 2008). Since the prostate gland is most susceptible to environmental insults during early development, it is reasonable to predict that prostate stem and early stage progenitor cells may be the primary targets of estrogenic exposures throughout life (Hu et al., 2011). Although there is accumulating evidence to suggest a central role for estrogens in prostate cancer, direct evidence that estrogens initiate prostate cancer in humans has been elusive. A rising E_2:T ratio in aging men (Mollard et al., 2000), association of estrogen metabolizing gene polymorphisms

and elevated urine hydroxy-estrone ratios with a higher prostate cancer risk (Kuiper et al., 1998a, Lemmen et al., 2004), a progressive increase in aromatase expression in prostate cancers upon advancement to metastatic disease (Song et al., 2002), and marked alterations in ER expression with cancer progression (Lowsley, 1912, Steiner and Pound, 2003) support the hypothesis that estrogens are involved in the etiology and progression of this disease. Further, tissue recombinant experiments using pre-initiated, human prostate BPH-1 cells have shown that elevated E2 and testosterone can promote these cells into invasive cancers (Wang et al., 2001, Ricke et al., 2006). Multiple studies over the past several decades using animal models have provided strong evidence of a carcinogenic role for estrogens in the prostate (Fouse et al., 2008, Henderson et al., 1982, Henderson et al., 1988, Walsh et al., 2005), but whether this is directly applicable to the human prostate had not been clarified.

Using the human prostasphere model described above, our laboratory recently discovered (Hu et al., 2011) that although negative for AR mRNA and protein, normal human prostate stem/ progenitor cells express robust levels of all known ERs, including ERα, ERβ, and GPR30 (Figure 3). Of note, the ER mRNA expression levels were markedly higher in normal prostate progenitor cells relative to the androgen-dependent prostate cancer cell line LNCaP and more closely resembled the steroid receptor profiles of the androgen-independent cancer lines PC-3 and DU145 with elevated ER expression, minimal progesterone receptor (PR), and no AR mRNA (Hu et al., 2011). Importantly, normal human prostaspheres exhibited a proliferative response to 1 nM 17β-estadiol resulting in increased sphere numbers and size at day 7 of culture. New studies using side-population FACS analysis of primary prostate epithelial cell cultures (Hoechst 33342 exclusion with and without verapamil) show a dose-dependent increase in stem cell numbers after 4 days of culture in 10-1000 nM E2 (Figure 5A). Together, these results demonstrate that normal human prostate progenitor cells are responsive to estrogens with increased rates of self-renewal, implicating them as direct estrogen targets.

Using the prostate *in vitro* stem cell assays described above, we are currently testing several EDCs with potential estrogenic action to determine if they too may be capable of affecting prostate stem cell self-renewal and/or differentiation capability. Treatment of primary prostate epithelial cells with 10 nM BPA increased the percentage of side population of prostate stem/progenitor cells (Figure 5B) similar to the E2 exposures. Dioxin (100 ng/ml) markedly increased side population numbers in a 2-D prostate epithelial cell cultures indicating a stimulation of stem cell self-renewal (Figure 5C). In contrast, 3-D culture of prostate epithelial cells in sodium arsenite markedly decreased the prostasphere number and size in a dose-

dependent manner (0.5, 5, 50 μM) (Figure 5D). Although preliminary, these examples suggest that estrogens and EDCs with estrogenic-type actions may have diverse effects on prostate stem/progenitor cells self-renewal, perhaps mediated through divergent ERs and other molecular signaling pathways. Since stem cell alterations are long lasting, such perturbations may contribute to prostate cancer risk throughout life.

To address the carcinogenic potential of estrogens on normal human prostate epithelial cells, we developed an experimental *in vivo* chimeric prostate model using normal human prostate progenitor cells from prostaspheres recombined with rat UGM engrafted under the renal capsule of nude mice (Hu et al., 2011). After one month of growth, the chimeric grafts formed normal prostate-like tissue with human epithelium that expressed CK8/18, p63, CK14, AR, PSA and human nuclear antigen (Figure 6A,B,C) (Hu et al., 2011). Subsequent exposure of the host mice to elevated E2 levels in an androgen-supported milieu was capable of driving multiple prostate lesions including epithelial hyperplasia, squamous metaplasia and initiation of prostate carcinogenesis with progression to locally invasive adenocarcinoma (Figure 6D,E,F) (Hu et al., 2011). Thus, by starting with human prostate stem/progenitor cells from young, disease-free organ donors, our study demonstrates for the first time that E2 is sufficient to initiate human prostate transformation and promote adenocarcinoma to a locally invasive phenotype. Support that estrogens are the culprit steroids in prostatic hormonal carcinogenesis comes from control grafts without T+E2 or with T implants alone that showed no evidence of prostate pathology after 3 months of growth (Hu et al., 2011). Together with the *in vitro* prostasphere data, our findings raise the intriguing possibility that stem and early progenitor cell populations in human prostate tissues might be susceptible targets of elevated E2 during the induction of hormonal carcinogenesis in aging males. This is particularly appealing in light of the recent evidence that transformation of prostate stem cells is sufficient for prostate cancer initiation in rodent and human models (Goldstein et al., 2010, Lawson et al., 2010).

In the above *in vivo* estrogen-initiated carcinogenesis model, the total lesion incidence in the chimeric grafts over 4 months was 43% epithelial hyperplasia, 31% high grade PIN and 11% adenocarcinoma (Hu et al., 2011). The relatively low cancer incidence in this novel system will permit the direct assessment of whether EDCs exposure are capable of increasing susceptibility of estrogen-induced PIN lesions and prostate cancer in human prostate tissue. Thus, through evaluation from both *in vitro* and *in vivo* assays, the new information gained will be of high value to the medical and regulatory communities in terms of providing strong and compelling evidence for negative effects of EDCs in humans. In addition, these models can be

used as a basis for studies in other target organs and contribute to long-term growth in the research enterprise on endocrine disruptors. If cancers are seeded by transformed stem cells as the stem cell theory for cancer development posits, an increased number of stem and progenitor cells in response to chronic and/or elevated estrogens and EDCs would increase prostate cancer risk by the shear presence of more cells available for transformation. Furthermore, it is possible that elevated estrogens and EDC exposures, acting through ER signaling pathways in the adult prostate progenitor cells, may directly reprogram or transform these cells, thus rendering them with tumor initiating capacity. Evidence in support of this comes from our studies in rodent models, where developmental estrogen exposures reprogram the prostate, leading to increased basal cell numbers and differentiation defects of the adult epithelium that predispose to dysplasia (Prins et al., 2001). Recent studies indicate that epigenetic mechanisms underlie developmental reprogramming of end organs by estrogens and EDCs (Ho et al., 2006, Newbold et al., 2006) and ongoing studies in our laboratories are underway to investigate epigenetic modifications in the human prostate stem/progenitor cells. In summary, studies using the novel human model systems described herein have the potential to provide direct evidence for an effect of early-life EDCs exposures on adult human prostate health and disease.

Figure legends

Figure 1. Isolation of prostate stem/progenitor cells from primary human prostate epithelial cells. (**A**) Human primary prostate epithelial cells (PrEC) in 2-D culture. PrEC contains a small number of prostate stem/progenitor cells at about 0.2~1%. (**B**) In a 3-D culture system, only the prostate stem/progenitor cells can self-renew and grow into a spheroid structure called prostasphere. Prostate stem/progenitor cells start to form visible prostaspheres (30~40 μm) on day 4. (**C**) By day 7, the sizes of prostaspheres reach a diameter of ~80 μm. (**D**) By day 10, prostaspheres grew bigger than 100 μm in diameter, cells locate in the center differentiate and form a double layer prostasphere. Bar=50 μm. (Hu et al, 2011)

Figure 2. Hoechst 33342 dye efflux fluorescence activated flow cytometry analysis reveals a side population gated as R1 in human prostate epithelial cells. Prostate epithelial cells were stained with 5 μg/ml of Hoechst 33343 either in the absence (**Left**) or presence (**Right**) of 50μM of verapamil hydrochloride, an ABCG2 inhibitor. Windows for the side-population are determined by comparison of cells without and with verapamil with each FACS analysis.

Figure 3. Day 7 prostasphere (PS) cells express prostate stem/progenitor cell markers and ERs. Immunofluorescent labeling of day 7 PS showed majority of PS cells are positive for membrane-associated CD117, TROP2, CD49f and ABCG2 (DAPI blue counterstain), confirms their stem/early progenitor cell characteristics. PS cells also express robust levels of all known ERs, including ERα and ERβ. The Bar=50 μm. (Hu et al, 2011)

Figure 4. In vitro differentiation of day 7 normal prostaspheres driven by 100 nM all-*trans* retinoic acid (RA) treatment. Prostaspheres form clear double layer structures after 7 days treatment with 100 nM RA (right) as compared to vehicle-treated spheres cultured in parallel as observed by brightfield microscopy (Top panel). Differentiation of prostasphere cells was indicated by the increased stainings of CK18 (2nd panel) and HOXB13 (3rd panel) as well as the decreased staining of p63 (bottom panel). Bar=50 μm.

Figure 5. Effects of E2, BPA, arsenite and dioxin on prostate stem/progenitor cell proliferation evaluated by side population analysis and prostasphere assay. (A) E2 (10 to 1000 nM) treatment increased the percentage of side population cells (R1) at all doses measured by Hoechst 33342 dye efflux fluorescence activated flow cytometry analysis. Prostate epithelial cells were stained with 5 μg/ml of Hoechst 33343 either in the absence (Graphs shown) or presence (not shown) of 50μM of verapamil hydrochloride before analysis. **(B)** BPA (10 nM) treatment also increased the percentage of side population cells (R1). **(C)** Dioxin (100 ng/ml) treatment increased the percentage of side population cells. **(D)** Sodium arsenite (0.5, 5, 50 μM) treatment decreased both the number and size of formed day 7 prostaspheres. Prostaspheres were grown for 7 days in a 3-D matrigel culture in the absence and presence of sodium arsenite, prostasphere numbers and sizes (40-80 μm, >80 μm diameter) were measured.

Figure 6. Characterization of chimeric prostate tissue from normal human prostate progenitor cells and prostate cancer in chimeric grafted tissue induced by T+E2. (A) H&E staining of a 1-month graft shows normal glandular structure with prostate histology (Graft inset). **(B)** Immunofluorescent staining with CK18 (red), CK14 (green) and **(C)** PSA (red) confirms differentiation of cells into prostate basal and luminal epithelial cells. **(D)** H&E section of graft exposed to T+E2 for 2 months reveals neoplastic epithelium with enlarged nuclei and

prominent nucleoli and their local invasion into the underlying stromal region (Graft inset). **(E)** Immunofluorescent staining with CK18 (red) and CK14 (green) confirms the local invasion of neoplastic cells into the stroma and the missing of CK14 positive basal cells. **(F)** Immunolabeling for PSA (red) confirms the human identity of the cancerous ducts and infiltrating cells in the grafted tissue. DAPI blue counterstain. Bar=50 µm. (Hu et al, 2011)

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