

Wilms' Tumor Blastemal Stem Cells Dedifferentiate to Propagate the Tumor Bulk

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SUMMARY

An open question remains in cancer stem cell (CSC) biology whether CSCs are by definition at the top of the differentiation hierarchy of the tumor. Wilms' tumor (WT), composed of blastema and differentiated renal elements resembling the nephrogenic zone of the developing kidney, is a valuable model for studying this question because early kidney differentiation is well characterized. WT neural cell adhesion molecule 1-positive (NCAM1⁺) aldehyde dehydrogenase 1-positive (ALDH1⁺) CSCs have been recently isolated and shown to harbor early renal progenitor traits. Herein, by generating pure blastema WT xenografts, composed solely of cells expressing the renal developmental markers SIX2 and NCAM1, we surprisingly show that sorted ALDH1⁺ WT CSCs do not correspond to earliest renal stem cells. Rather, gene expression and proteomic comparative analyses disclose a cell type skewed more toward epithelial differentiation than the bulk of the blastema. Thus, WT CSCs are likely to dedifferentiate to propagate WT blastema.

INTRODUCTION

The cancer stem cell (CSC) model suggests that whereas most tumor cells are destined to differentiate, albeit aberrantly, a small subset of tumor cells, termed CSCs, actively sustain and propagate the tumor. Although CSCs are considered to be at the top of the differentiation hierarchy of the tumor, this has not been examined in human tumors. Wilms' tumor (WT), a prototype of differentiation failure in human cancer, shares the histology of the fetal kidney, including blastema, stroma, and differentiating tubular epithelium (Shukrun et al., 2014; Dekel et al., 2006; Pode-Shakked and Dekel, 2011; Rivera and Haber, 2005). Because normal renal differentiation is well characterized, WT represents an invaluable model for establishing the position of CSC with respect to this differentiation cascade.

Kidney development is initiated when the Wolffian duct sends off a dorsal branch, the ureteric bud (UB), to invade the metanephric mesenchyme (MM) (Pleniceanu et al., 2010). The UB receives MM-derived signals to undergo branching morphogenesis and develop into the collecting system. Reciprocally, a fraction of the MM, termed cap mesenchyme (CM), receives signals from the UB tips, facilitating its survival, proliferation, and mesenchymal-to-epithelial transition (MET) into nephron epithelia.

Sequential steps during renal MET have been characterized by specific gene expression and signaling pathway activity. This process is accompanied by gradual loss of renal developmental factors and mesenchymal markers (e.g., SIX2, CITED1, and vimentin) and acquisition of epithelial proteins (e.g., E-Cadherin) (Cirio et al., 2014; Metsuyanin et al., 2009; Self et al., 2006). The CM has been recently shown to consist of true stem cells, capable of self-renewing and differentiating toward different types of nephron epithelia (Cirio et al., 2014; Kobayashi et al., 2008).

We have recently uncovered the identity of human WT CSCs. WT CSCs were isolated from trilineage WT (composed of blastema, stroma, and tubular epithelium) propagated in mice and characterized by high expression levels of neural cell adhesion molecule 1 (NCAM1) and aldehyde dehydrogenase 1 (ALDH1) enzymatic activity (Pode-Shakked et al., 2013). These cells possessed both self-renewal and multilineage differentiation capacities, in line with the CSC definition. Immunohistochemical (IHC) analysis of WTs revealed that the CSC population was exclusively localized and scattered within the NCAM1-expressing blastema (Pode-Shakked et al., 2013). The WT blastema, suggested to resemble the CM harboring multipotent embryonic renal stem cells, is classically regarded as a homogeneous unit. Thus, whereas our analysis demonstrated WT CSCs to overexpress the renal



developmental gene set (i.e., *SIX2*, *OSR1*, *PAX2*, and *SALL1*), compatible with early renal progenitor cells, it was confounded by the presence of mature elements, and the precise developmental phenotype in regard to renal lineage differentiation remained elusive.

Therefore, to pinpoint the exact stage of the WT CSC in respect to embryonic renal stem cell differentiation, high-resolution analysis of a pure blastema population is required. Here, we created an *in vivo* model of pure blastema WT via serial propagation of human WT xenograft (Xn). This allowed us to show that the WT blastema is a heterogeneous-differentiating cell population that follows the renal developmental MET axis. Within this differentiation gradient of WT blastema, we show that WT CSCs are committed epithelial progenitors, and not the earliest mesenchymal renal stem cells, the presumable WT cell of origin (Li et al., 2002). Hence, WT CSCs must possess both dedifferentiation capacity forming less-differentiated, *SIX2*-high blastemal cells, as well as epithelial differentiation capability, as previously shown for *NCAM1*⁺*ALDH1*⁺ WT CSCs (Pode-Shakked et al., 2013). Dedifferentiation of WT CSCs into early mesodermal cells might also explain the presence of heterologous mesodermal elements such as muscle and bone seen in some WTs (Royer-Pokora et al., 2010).

RESULTS

Generation of Pure Blastema WT Xns

Previous work in our lab demonstrated that whereas WT blastema is lost upon *in vitro* propagation, generation of WT Xn preserves and even expands the blastema *in vivo* (Dekel et al., 2006; Metsuyanin et al., 2009). Here, we show that late-generation (passages 10–15) Xns are composed solely of homogeneous-appearing sheets of blastema cells (Figure 1A). Recently, utilizing low-passage WT Xns (passages 1–5), which maintain most of the primary tumor's properties (i.e., gene expression and trilineage histology), we isolated CSCs that exclusively initiate and sustain the tumor *in vivo* (Dekel et al., 2008). These *NCAM1*⁺*ALDH1*⁺ WT CSCs were shown to harbor renal stem cell characteristics compared to all other tumor components (Pode-Shakked et al., 2013). To further analyze late-generation Xns, we examined *NCAM1* expression in comparison with primary human WT and early WT Xns. IHC revealed *NCAM1* enrichment along serial passages of the tumor in mice (Figures 1B and 1C, left panel). Flow cytometry analysis performed on late Xns demonstrated that 100% of the cells express *NCAM1* (Figure S1A available online). In addition, IHC showed these pure blastema WT Xn cells to uniformly express *SIX2*. (Figure 1C, middle panel). Altogether, pure blastema WT Xns

are solely composed of *NCAM1*⁺*SIX2*⁺ cells, indicating their primitive renal nature.

The Tumorigenic Potential of Pure Blastema WT Xns Is Maintained Solely by the *ALDH1*⁺ Cell Population

Establishment of pure blastema Xns afforded the opportunity to sort single *ALDH1*⁺ cells and determine whether they exclusively function as CSCs and investigate their lineage relationship to all other non-CSC blastema cells. Localization of *ALDH1* was initially determined in the pure blastema model using IHC. Scattered *ALDH1*⁺ cells were localized within the *SIX2*⁺ blastema (Figure 1C, right panel; Figure S1B). Late Xn cells were then sorted according to *ALDH1* activity with high efficiency as shown via quantitative real-time PCR analysis (Figure S2A). Limiting-dilution xenotransplantation experiments revealed that only *ALDH1*⁺ cells have the capacity to form tumors. Moreover, as few as 200 cells were sufficient to form new WT Xns, demonstrating the high tumorigenic potential of *ALDH1*⁺ cells (Figure 2A). Thus, as in early passages, in these late passages, the ability to sustain and propagate the tumor is maintained solely by *ALDH1*⁺ cells. Because resistance to conventional radio- and chemotherapies is a trait of CSCs in several malignancies (Bonnet and Dick, 1997; Reya et al., 2001), we examined the *in vitro* response of *ALDH1*⁺ WT cells to radiation. Following exposure of WT Xn cells to ionizing radiation, an increase in *ALDH1*⁺ cells was noted (Figure 2B). Moreover, the percentage of *ALDH1*⁺ cells correlated with exposure time and radiation intensity in a dose-dependent manner (Figure 2C). Hence, *ALDH1*⁺ WT CSCs demonstrate increased resistance to conventional radiation treatment. Taken together, these results indicate that *ALDH1*⁺ cells function as CSCs in pure blastema Xns.

Analysis of WT CSC Fraction in Relation with Embryonic Renal Differentiation Reveals Developmental Heterogeneity in WT Blastema

We have previously shown that early WT Xns derived from sorted *NCAM1*⁺*ALDH1*⁺ CSCs harbor a higher percentage of *ALDH1*⁺ cells compared to tumors derived from unsorted cells (USs). In order to determine whether this trait is retained in pure blastema WT Xns, we compared *ALDH1* expression between late Xns derived from *ALDH1*⁺ sorted cells and those derived from USs. Fluorescence-activated cell sorting (FACS) analysis revealed that the former are enriched for *ALDH1*⁺ CSCs (21.2% versus 5.6%, respectively) (Figures 3A, 3B, and S2B). Next, we performed global gene expression and proteomic analyses on these CSC-enriched (CSC-En) tumors in comparison with tumors derived from USs from the same tumor source (Figures 3C, 3D, and Table S1, respectively). We performed a microarray gene expression analysis comparing three samples: (1) human fetal

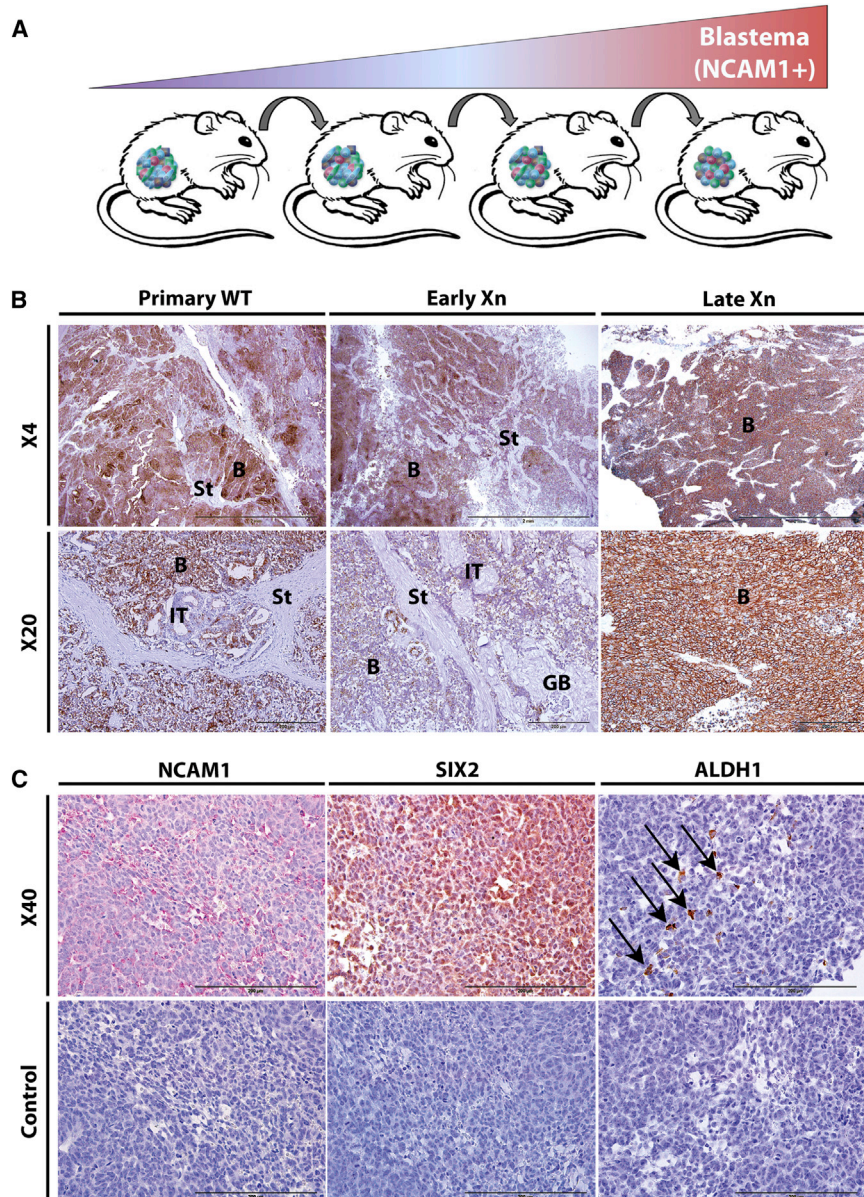


Figure 1. Generation of Pure Blastema WT Xns

(A) Serial propagation of human WT Xn in NOD-SCID mice results in enrichment of NCAM1⁺ WT blastema, at the expense of differentiated structures.

(B) IHC staining of pWTs, early-passage Xns, and late-passage Xns for NCAM1. Early passages show great histological similarity to their parent tumor (B, blastema; IT, immature tubule; St, stroma; GB, glomeruloid bodies) with minor enrichment for the blastema (NCAM1⁺) compartment. However, late-passage Xns are composed of 100% NCAM1⁺ blastemal cells and are devoid of differentiated elements. Scale bars, 2 mm (top) and 200 μm (bottom).

(C) IHC staining of pure blastema WT Xns for NCAM1 (right), SIX2 (middle), and ALDH1 (left). All the tumor cells are NCAM1⁺SIX2⁺, whereas ALDH1⁺ cells are scattered within these blastemal sheets. Scale bars, 200 μm. See also [Figure S1](#).

kidney (hFK), (2) US tumors, and (3) CSC-En tumors. First, we performed hierarchical clustering in order to characterize the three samples with respect to the renal developmental signature, as manifested by the expression of several renal developmental genes (i.e., *SIX2*, *SALL1*, *OSR1*, *CITED1*, and *PAX2*). The results demonstrated genetic proximity between the two Xn samples, both expressing high levels of the aforementioned genes, compared to hFK, confirming that the pure blastema Xns harbor properties of primitive renal tissue. Interestingly, when comparing the two Xn samples, we found that CSC-En Xns expressed lower levels of the renal developmental genes ([Figure 3C](#)). We next carried out a quantitative real-

time PCR analysis comparing the two Xn samples. This comparison validated the lower expression levels of the renal developmental gene set in the CSC-En sample. Importantly, CSC-En tumors showed higher expression of stemness genes (i.e., *NANOG*, *BMI1*, and *EZH2*), supporting enrichment for WT CSCs ([Figure 3D](#)). In addition, proteomic analysis supported the gene expression data, demonstrating a relative epithelial phenotype (i.e., high E-Cadherin) of the CSC-En tumors compared to US tumors ([Table S1](#)). Concomitantly, CSC-En tumors show upregulation of stemness-associated signaling pathways at the protein level (e.g., nuclear factor-κB [NF-κB], Wnt, phosphatidylinositol 3-kinase [PI3K], and mammalian target

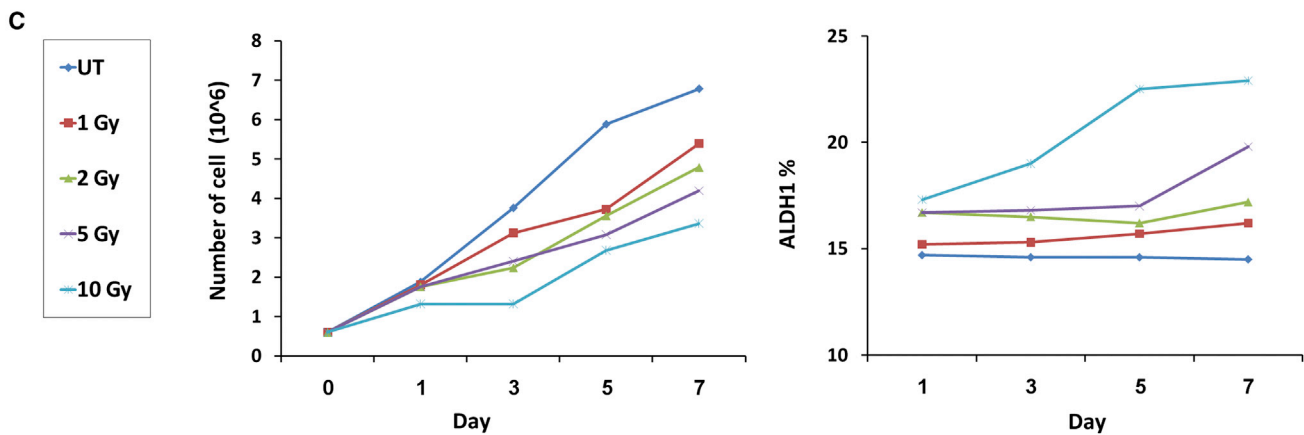
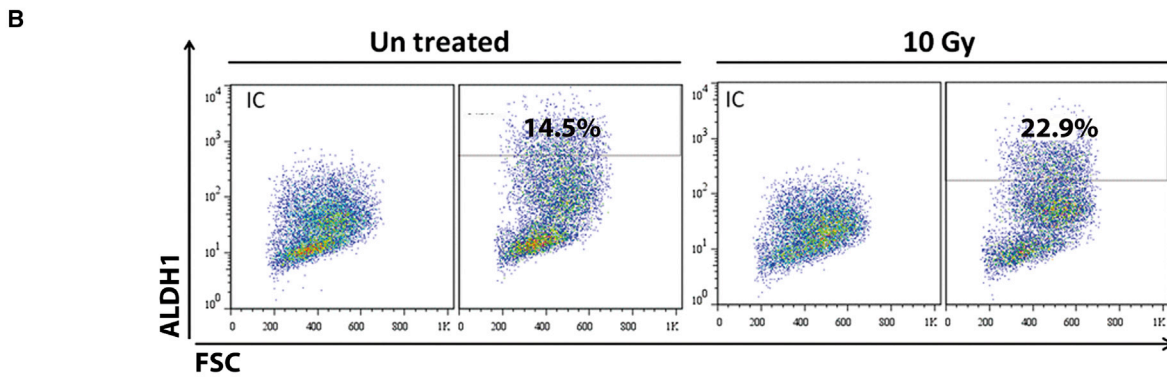
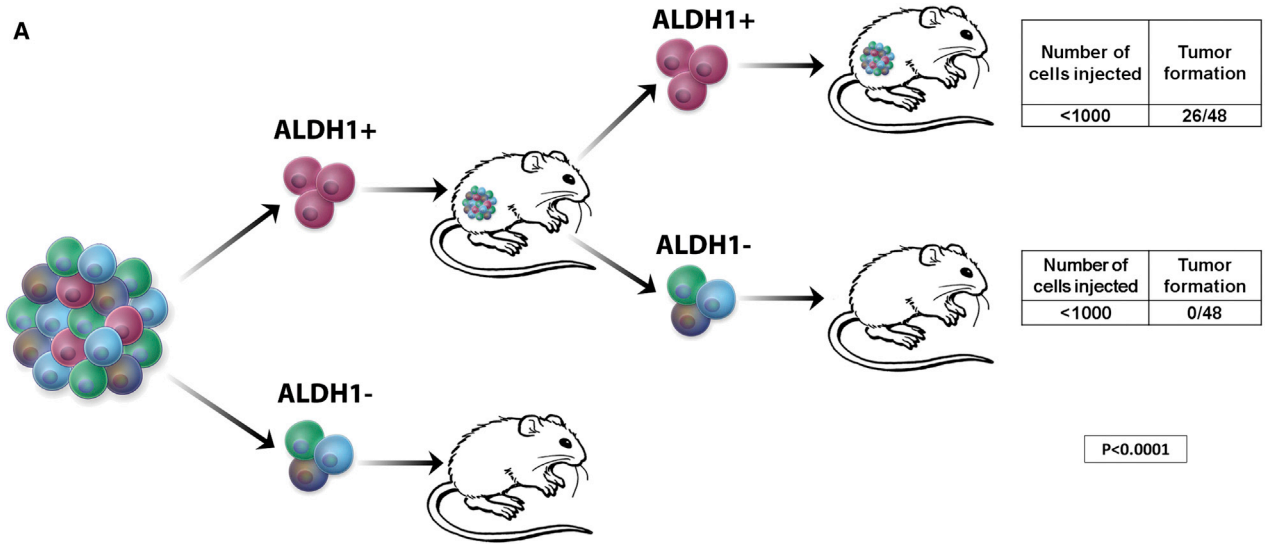


Figure 2. The Tumorigenic Potential of Pure Blastema WT Xns Is Maintained Solely by the ALDH1⁺ Cell Population

(A) Scheme shows that ALDH1⁺ WT cells retain their CSC phenotype in pure blastema Xns. Pure blastema Xns were sorted according to ALDH1 activity. ALDH1⁺ and ALDH1⁻ blastemal cells were then injected into mice. Only ALDH1⁺ cells were able to initiate tumors in all dilutions applied, whereas ALDH1⁻ cells failed to form tumors altogether. Following ALDH1⁺-derived tumor growth, sorting and injections of ALDH1⁺ and ALDH1⁻ cells were repeated with equivalent results, confirming that ALDH1⁺ cells maintain self-renewal capacity in pure blastema Xns.

(B) Representative FACS analysis of untreated Xn cells in comparison to Xn cells treated with ionizing radiation at an intensity of 10 Gy reveals enrichment of ALDH1⁺ population.

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of rapamycin [mTOR]) (Table S1). Thus, pure blastema WT Xns, enriched for ALDH1⁺ CSCs, demonstrate a more epithelial phenotype than their US-derived counterparts, while maintaining their stemness. We next determined whether culture conditions of pure blastema Xn cells can recapitulate in vivo findings. We employed low-attachment conditions to grow CSC-En tumor spheres (Yano et al., 2013) and interrogated their phenotype by comparison to adherent culture. Tumor spheres demonstrated high ALDH1 expression, accompanied by high expression of stemness genes (e.g., *OCT4*, *NANOG*, and Polycomb genes) and skewing toward a more epithelial phenotype (i.e., lower *SIX2*, *OSR1*, *CITED1*, *vimentin*, and higher *E-Cadherin* and *EpCAM* levels) (Figure 3E). Thus, altering culture conditions to enrich for CSCs can result in a relative renal MET, mirroring the in vivo state.

Global Gene Analysis Reveals that WT CSC Is Arrested at a Renal Progenitor State, Committed to Epithelial Differentiation

We next sought to characterize the molecular profile of the ALDH1⁺ CSCs. For this purpose, we performed a microarray gene expression analysis comparing three samples: (1) sorted ALDH1⁺ cells, (2) sorted ALDH1⁻ cells, and (3) USs. This comparison revealed a unique gene expression profile of the ALDH1⁺ CSCs that included high levels of stemness genes (e.g., *OCT4*, *SHH*, *LIN28A*, and *KLF4*) and relatively high levels of epithelial markers (e.g., Keratin 6c, Keratin 33a, Keratin 35, Claudin 7, Claudin 9, and Claudin 13) alongside lower levels of mesenchymal markers (e.g., *vimentin*, *SNAI2*, and *MEST*) (Figures 4A and S3). Importantly, ALDH1⁺ cells demonstrated lower levels of proliferation-related genes (e.g., *PCNA*, *CCNB1*, and *E2F1*) compared to ALDH1⁻ cells, suggesting a more quiescent phenotype of this population (Figure 4A). In order to validate the microarray data, quantitative real-time PCR was performed on sorted ALDH1⁺ and ALDH1⁻ cells derived from three different pure blastema WT Xn sources. Characterization of these fractions demonstrated that indeed ALDH1⁺ cells express lower levels of renal developmental genes (Figure 4B). Moreover, higher *E-Cadherin* and lower *vimentin* expression in ALDH1⁺ cells was noted in comparison to ALDH1⁻ cells (Figure 4B). In order to further evaluate the proliferative state of the different factions within the pure blastema WT Xns, we carried out flow cytometry cell-cycle analysis. In accordance with the microarray data, the ALDH1⁺ fraction demonstrated a higher percentage of cells in the G0/G1 phase

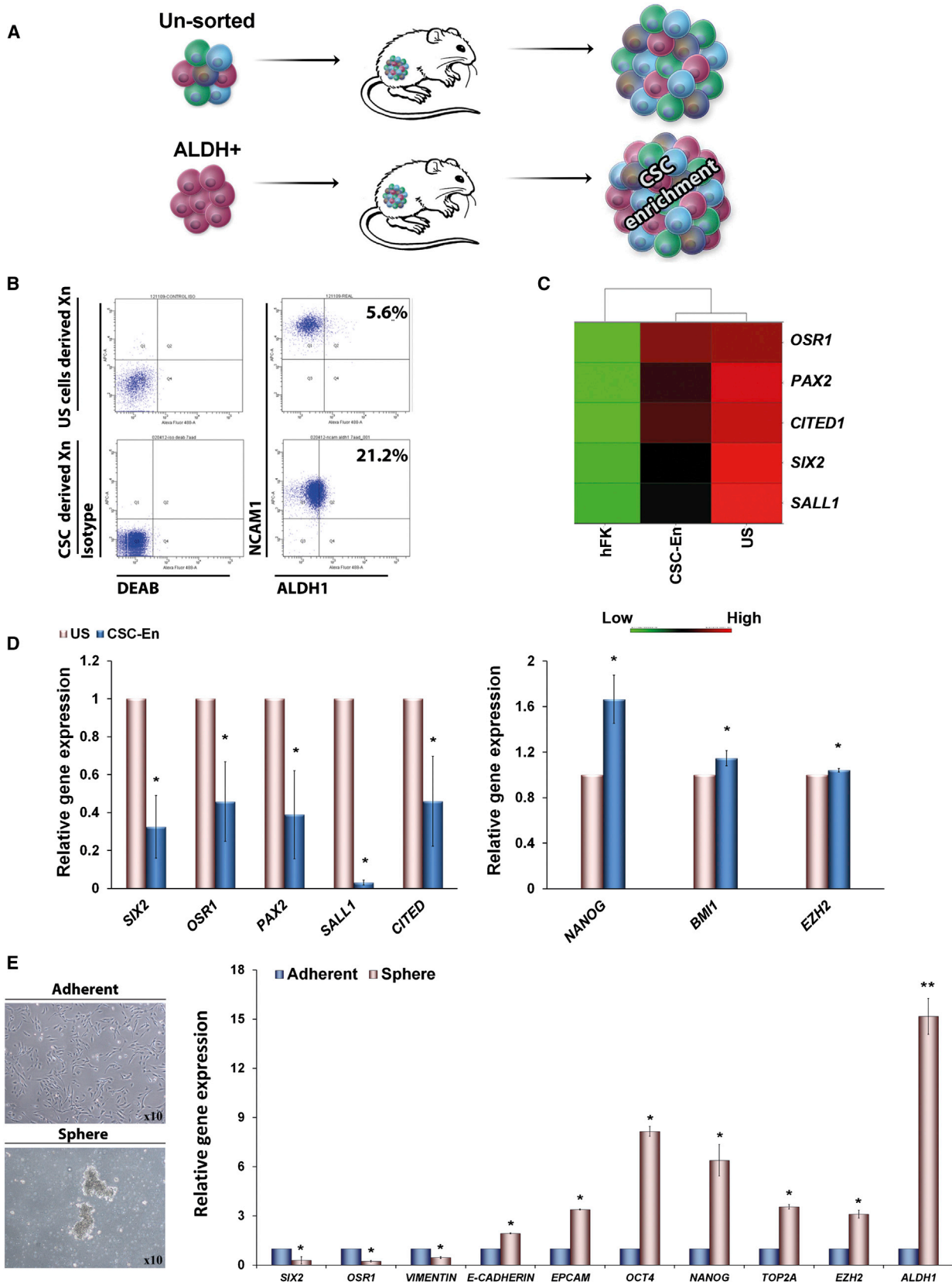
compared to USs (87.4% and 75.8%, respectively) (Figure S4). We next characterized ALDH1⁺ cells at the protein level. We performed western blot analysis of renal MET protein expression comparing four samples: (1) sorted ALDH1⁺ Xn cells, (2) sorted ALDH1⁻ Xn cells, (3) unsorted Xn cells, and (4) primary WT (pWT). The results demonstrated lower expression of *SIX2* and higher expression of *E-Cadherin* in ALDH1⁺ cells (Figure 4C). Interestingly, pWT presented higher *E-Cadherin* levels and lower *vimentin* and *SIX2* levels compared to the Xn samples, emphasizing the mesenchymal, undifferentiated nature of the blastema-pure tumors. In addition, immunofluorescence (IF) staining of sorted ALDH1⁺ and ALDH1⁻ WT Xn cells for *SIX2* and the epithelial markers *E-Cadherin* and cyto-keratin (MNF 116) showed distinct staining patterns. ALDH1⁺ cells presented significantly lower expression of *SIX2* (Figure 4D, left) and higher expression of epithelial markers (Figure 4D, middle and right). Furthermore, in order to demonstrate in vitro dedifferentiation capacity of the ALDH1⁺ sorted fraction, cells were grown in culture for 10 days. We observed the acquisition of a mesenchymal phenotype, as manifested by upregulation of *vimentin* alongside downregulation of ALDH1 expression and the epithelial markers *E-Cadherin* and *EpCAM* (Figure 4E). Taken together, these results suggest that the WT CSCs are not the least differentiated cells along the renal developmental MET axis but, rather, are arrested at a renal progenitor state, committed to epithelial differentiation. Thus, the WT CSCs are likely to dedifferentiate to propagate WT blastema.

DISCUSSION

Here, by comparing the WT CSCs with normal kidney development, we discover two surprising fundamental concepts. First, in terms of lineage hierarchy, the CSC may not correspond to the earliest normal stem cell but rather to a more differentiated progeny. Second, heterogeneity that mirrors renal stem cell differentiation is present in the homogeneous-appearing WT blastema. Our earlier observations (Pode-Shakked et al., 2013) disclosed high similarity between WT CSCs and early renal stem cells. These observations were based on propagation of trilineage human WT, in which the relationship of the CSC to tumor bulk includes a comparison to blastema and also to mature elements, placing the CSC as an undifferentiated renal cell type. Our ability to examine pure blastema Xns enabled

(C) Summary of ionizing radiation effect on proliferation and ALDH1 expression of Xn cells. Left view shows that ionizing radiation inhibits cell growth in a dose- and time-dependent manner. Right view shows that exposure of WT Xn cells to ionizing radiation enriches for the ALDH1⁺ CSC population in a dose- and time-dependent manner.

See also Figure S2.



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stringent comparison of WT CSCs to the undifferentiated tumor bulk and the unraveling of the findings. Indeed, the importance of the fine-tuning of our system is manifested when comparing WT CSCs to parental tissues (i.e., hFK and pWT), disclosing a relatively undifferentiated phenotype. This precise understanding of the relation between the WT CSC and embryonic renal differentiation affords insights from both oncologic and developmental aspects. Multiple studies have highlighted the relationship between CSCs and their differentiated progeny (Reya et al., 2001), including WT CSCs, and their ability to generate tubular structures and glomeruloid bodies (Little, 2005; Pode-Shakked and Dekel, 2011; Pode-Shakked et al., 2013). Nevertheless, the dedifferentiated progeny arising from ALDH1⁺ WT CSCs, represented by the bulk of the blastema and characterized by higher *SIX2* and lower *E-Cadherin*, illustrates mechanisms for WT and progression. Interestingly, partial reprogramming by pluripotent stemness factors has been recently implicated in generation of a Wilms'-like tumor in vivo (Ohnishi et al., 2014). Hypothetically, dedifferentiation of WT CSCs may also account for the nonrenal mesenchymal elements observed in WT. This notion is strengthened by the fact that developmental lineages are already specified in the MM, and no common nephron epithelial-stromal progenitor cell has been documented (Brown et al., 2013; Kobayashi et al., 2008; Pleniceanu et al., 2010; Self et al., 2006). Our work may suggest a revision to the concept that the earliest transformed renal stem cell capable of giving rise to differentiated progeny is the tumor-initiating cell. Alternatively, the WT cell of origin and WT CSCs responsible for tumor propagation may not be the same cell. Resolution of this matter utilizing a transgenic animal model of WT generation that employs specific CRE drivers of renal stem cells (e.g., *SIX2*) might be complicated because WT CSCs express

some levels of *SIX2* and *WT1*. Finally, from the developmental aspect, it remains to be determined how NCAM1⁺ human nephron progenitors isolated from the hFK and representing more committed epithelial progenitors than uninduced MM (Harari-Steinberg et al., 2013) are relevant for WT once transformed.

From a more practical aspect, the unequivocal limiting-dilution xenotransplantation data, illustrating ALDH1⁺ WT CSCs as critical for continued propagation of WT blastema Xns, highlight the WT CSC as a therapeutic target. We previously suggested anti-NCAM1-targeted therapy as a useful means for WT eradication (Pode-Shakked et al., 2013). Clearly, the NCAM1 expression domain exceeds that of the WT CSC. In this regard, our genomic and proteomic data disclose several specific pathways (e.g., NF- κ B, Wnt, PI3K, and mTOR) previously implicated in stemness acquisition (Armstrong et al., 2006; Huang et al., 2012; Katoh and Katoh, 2007; Shostak and Chariot, 2011) as highly operative in WT CSCs. Thus, global analysis not only sheds light on the WT CSC as possessing enhanced stemness alongside a more differentiated renal phenotype but also pinpoints interventions such as mTOR and epidermal growth factor receptor inhibition that may prove beneficial in WT CSC eradication.

EXPERIMENTAL PROCEDURES

Detailed [Experimental Procedures](#) are provided in the [Supplemental Experimental Procedures](#).

pWT Samples

pWT samples were obtained from patients with WT within 1 hr of surgery from both Sheba Medical Center and Hadassah-Ein Kerem hospital. All studies were approved by the local ethics committee,

Figure 3. Analysis of WT CSC Fraction in Relation with Renal MET Axis Reveals Developmental Heterogeneity in WT Blastema

(A) Scheme presents WT Xns derived from ALDH1⁺ sorted cells (bottom) that show enrichment in ALDH1⁺ CSCs in comparison with pure blastema WT Xns derived from USs (top).

(B) Representative FACS analyses of WT Xns derived from either unsorted WT cells (top) or ALDH1⁺ WT cells (bottom) showing enrichment in ALDH1⁺ WT CSCs in the latter.

(C) Gene heatmap of pure blastema CSC-En tumors in comparison to US-derived Xn and hFK. This analysis revealed that the CSC-En Xn and, to an even greater extent, the Xn derived from USs are enriched for the earliest renal developmental gene set in comparison to the hFK. (D) Validation via quantitative real-time PCR of renal developmental genes (i.e., *WT1*, *SIX2*, *SALL1*, *OSR1*, *CITED1*, and *PAX2*) (left) and stemness genes (i.e., *NANOG*, *BMI1*, and *EZH2*) (right) in unsorted and CSC-En Xns. For quantitative real-time PCR analyses, the values for unsorted-derived pure blastema WT Xn cells were used to normalize (therefore equaling one [1]), and all other values were calculated with respect to them. Results are presented as the mean \pm SEM of five separate experiments obtained from three different patients. * $p < 0.05$.

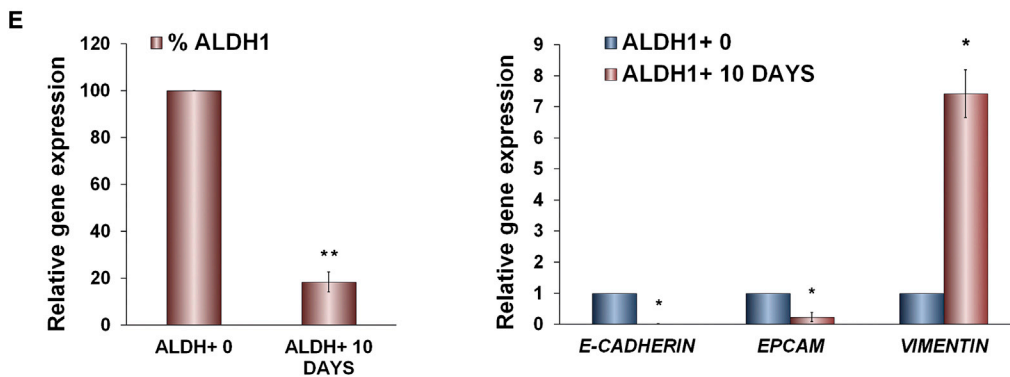
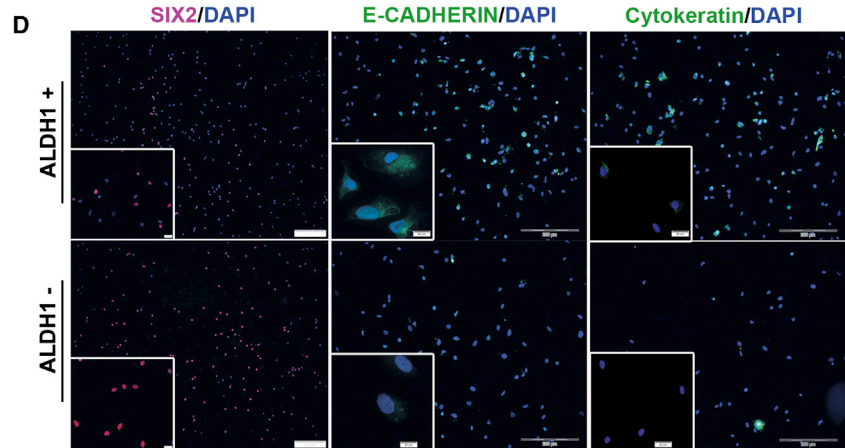
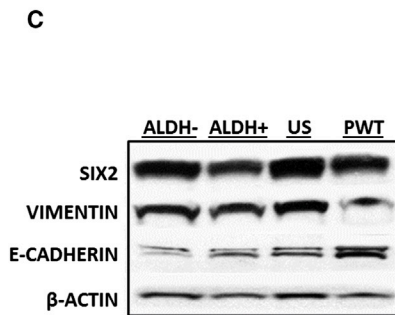
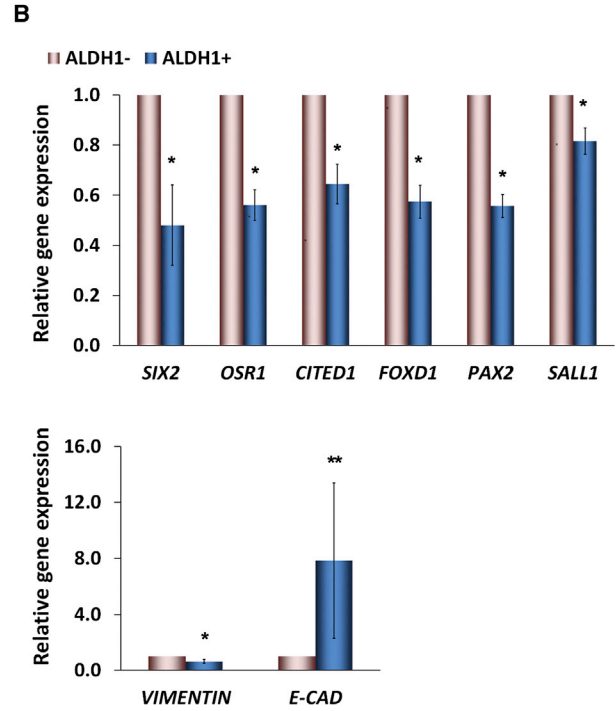
(E) Unsorted Xn cells were grown in two different culture conditions (images by light microscopy are shown on the left): adherent cells (top), and sphere-forming cells, grown in low-attachment conditions (bottom). The spheres demonstrated high *ALDH1* expression accompanied by high levels of stemness genes (i.e., *OCT4*, *NANOG*, *TOP2A*, and *EZH2*) along with a more epithelial phenotype (lower levels of *SIX2*, *OSR1*, *CITED1*, and *vimentin* and higher levels of *E-Cadherin* and *EpCAM*). The values for adherent cells were used to normalize (therefore equaling one [1]), and other values were calculated with respect to them. Results are presented as the mean \pm SEM of three separate experiments. * $p < 0.05$; ** $p < 0.01$.

See also [Figure S2](#) and [Tables S1](#) and [S2](#).



A

Function	ID	Gene Symbol	ALDH1+ vs. ALDH1-
	11723713_at	<i>E2F1</i>	0.62
	11760225_at	<i>PLK1S1</i>	0.55
	11732997_a_at	<i>MCM6</i>	0.52
	11720971_at	<i>TOP2A</i>	0.50
Cell proliferation	11744169_a_at	<i>MYBL2</i>	0.45
	11759510_x_at	<i>MCM4</i>	0.44
	11723939_a_at	<i>CCNB1</i>	0.43
	11756666_x_at	<i>MCM2</i>	0.34
	11747230_a_at	<i>BUB1</i>	0.30
	11716103_a_at	<i>PCNA</i>	0.23
	11724407_s_at	<i>CCNE1</i>	0.23
	11742996_a_at	<i>MCM3</i>	0.11
	11725907_at	<i>SIX2</i>	0.18
Renal patterning	11724865_at	<i>OSR1</i>	0.44
	11732702_a_at	<i>CITED1</i>	0.55
	11728563_at	<i>FOXD1</i>	0.49
	11728624_a_at	<i>PAX2</i>	0.70
	11738265_at	<i>SHH</i>	6.26
Stemness	11755599_x_at	<i>POU5F1</i>	1.65
	11725430_at	<i>LIN28A</i>	1.96
	11719633_a_at	<i>KLF4</i>	2.40
		11731342_at	<i>CLDN9</i>
	11739907_at	<i>CLDN16</i>	1.87
	11727492_at	<i>KRT6C</i>	5.45
	11738651_at	<i>KRT35</i>	4.84
MET/EMT	11715296_s_at	<i>KRT33A</i>	3.28
	11755943_x_at	<i>VIM</i>	0.63
	11744985_a_at	<i>FNDC3A</i>	0.53
	11744218_at	<i>SNAI2</i>	0.22
	11754365_s_at	<i>ROR2</i>	0.04
	11723029_x_at	<i>MEST</i>	0.23
	11716195_a_at	<i>ID1</i>	0.57



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and informed consent was given by the legal guardians of the patients involved according to the Declaration of Helsinki.

In Vivo Xn Formation

The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Sheba Medical Center. Initial WT xenografting to 5- to 8-week-old, female, nonobese diabetic severe combined immunodeficiency (NOD-SCID) mice was performed as previously described (Dekel et al., 2006). Late-passage Xns were formed by serial injections of approximately 10^6 dissociated cells from freshly retrieved WT Xns. Cells were injected in 100 μ l 1:1 serum-free medium/Matrigel (BD Biosciences). See also [Supplemental Experimental Procedures](#).

Statistical Analysis

Results are expressed as the mean \pm SEM, unless otherwise indicated. Statistical differences in gene expression between WT cell populations were evaluated using the nonparametric, one-sided sign test. Statistical differences between additional data groups were determined with Student's *t* test. For all statistical analyses, the level of significance was set as $p < 0.05$, unless otherwise indicated.

ACCESSION NUMBERS

The GEO accession number for the chromatin immunoprecipitation array data reported in this paper is GSE57269.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.05.013>.

AUTHOR CONTRIBUTIONS

B.D., R.S., and N.P.-S. designed the experiments. R.S. and S.P.-C. performed the in vivo experiments. R.S., E.P., E.V., Q.H., V.H., O.H.-S., and D.O. performed in vitro assays. B.D., J.J., R.S., and N.P.-S. analyzed the data. B.D., R.S., O.P., and N.P.-S. wrote the manuscript.

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Figure 4. Global Analysis Reveals a Distinct Profile of CSCs Congruent with MET and Not Uninduced Mesenchymal Progenitors

Molecular profile analyses of WT ALDH1⁺ CSCs.

(A) Microarray gene expression analysis of WT CSCs. A table is shown of gene expression fold change comparing sorted ALDH1⁺ and ALDH1⁻ cells. ALDH1⁺ CSCs demonstrate low levels of proliferation-related genes (e.g., *PCNA*, *CCNB1*, and *E2F1*), lower levels of renal developmental genes (e.g., *SIX2*, *OSR1*, and *CITED1*), high levels of stemness genes (e.g., *POU5F1*, *SHH*, *LIN28A*, and *KLF4*), high expression of epithelial markers (*E-Cadherin*, *Keratin 6c*, *Keratin 33a*, *Keratin 35*, *Claudin 7*, *Claudin 9*, and *Claudin 13*), and lower levels of mesenchymal markers (*vimentin*, *SNAI2*, and *MEST*).

(B) Quantitative real-time PCR demonstrating significantly reduced levels of renal-patterning genes (i.e., *SIX2*, *OSR1*, *CITED1*, *SALL1*, and *PAX2*) along with elevation of *E-Cadherin* (*E-CAD*) and decreased *vimentin* in ALDH1⁺ cells. The values for ALDH1⁻ WT cells were used to normalize (therefore equaling one [1]), and all other values were calculated with respect to them. Results are presented as the mean \pm SEM of five separate experiments obtained from three different patients. * $p < 0.05$; ** $p < 0.01$.

(C) Western blot analysis of renal MET protein expression in four samples: (1) ALDH1⁺ cells, (2) ALDH1⁻ cells, (3) USs, and (4) pWT cells. This analysis demonstrated lower expression of *SIX2* and higher expression of *E-Cadherin* in ALDH1⁺ in comparison with ALDH1⁻ WT cells. In addition, pWT cells present higher levels of *E-Cadherin* and lower levels of *vimentin* and *SIX2* in comparison to the Xn samples, emphasizing the mesenchymal nature of the blastema-pure tissues.

(D) IF staining of sorted ALDH1⁺ and ALDH1⁻ WT Xn cells for *SIX2* (left panel) and the epithelial markers: *E-Cadherin* (middle panel) and cytokeratin (right panel). A distinct staining pattern showing higher protein expression of the epithelial markers and lower expression of *SIX2* in ALDH1⁺ in comparison with ALDH1⁻ cells can be seen. Scale bars, 200 μ m.

(E) ALDH1⁺ sorted cells were grown for 10 days in culture. Gene expression comparison of noncultured cells (ALDH⁺ 0) and cultured cells (ALDH⁺ 10 days) revealed significant downregulation of *ALDH1* expression (left) and upregulation of *vimentin* alongside downregulation of the epithelial markers *E-Cadherin* and *EpCAM* (right), demonstrating plasticity of the ALDH1⁺ CSCs. The values for ALDH⁺ 0 cells were used to normalize (therefore equaling one [1]), and all other values were calculated with respect to them. Results are presented as the mean \pm SEM of three separate experiments. * $p < 0.05$; ** $p < 0.01$.

See also [Figures S3](#) and [S4](#) and [Table S3](#).



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