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Aldehyde dehydrogenase 3A1 associates with prostate tumorigenesis

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Background: Accumulating evidence demonstrates high levels of aldehyde dehydrogense (ALDH) activity in human cancer types, in part, because of its association with cancer stem cells. Whereas ALDH1A1 and ALDH7A1 isoforms were reported to associate with prostate tumorigenesis, whether other ALDH isoforms are associated with prostate cancer (PC) remains unclear.

Methods: ALDH3A1 expression was analysed in various PC cell lines. Xenograft tumours and 54 primary and metastatic PC tumours were stained using immunohistochemistry for ALDH3A1 expression.

Results: In comparison with the non-stem counterparts, a robust upregulation of ALDH3A1 was observed in DU145-derived PC stem cells (PCSCs). As DU145 PCSCs produced xenograft tumours with more advanced features compared with those derived from DU145 cells, higher levels of ALDH3A1 were detected in the former; a dramatic elevation of ALDH3A1 occurred in DU145 cell-derived lung metastasis compared with local xenograft tumours. Furthermore, while ALDH3A1 was not observed in prostate glands, ALDH3A1 was clearly present in PIN, and further increased in carcinomas. In comparison with the paired local carcinomas, ALDH3A1 was upregulated in lymph node metastatic tumours; the presence of ALDH3A1 in bone metastatic PC was also demonstrated.

Conclusions: We report here the association of ALDH3A1 with PC progression.

Prostate cancer (PC) is the most common cancer affecting men in the developed world (Williams and Powell, 2009). The disease progresses from intra-epithelial neoplasia (PIN) or *de novo*, locally invasive carcinoma to metastatic cancer (Ross, 2007; Moon *et al*, 2008). The most effective treatment for patients with metastatic PCs is androgen ablation, pioneered by Charles Huggins in 1941 (Rosenberg and Small, 2003; Ross, 2007). Whereas the treatment leads to a clinical response in 80% of patients, hormone refractory PC (HRPC) commonly arises. This type of cancer leads to a median survival time of ~1 year (Ross, 2007; Moon *et al*, 2008). As a result, metastatic PC remains incurable.

Although the mechanisms responsible for developing metastasis are poorly understood, accumulating evidence suggests that cancer stem cells (CSCs) are responsible for metastasis (Yang *et al*, 2008). The epithelial–mesenchymal transition (EMT) is widely regarded as a critical event for metastasis of epithelial tumours (Visvader and Lindeman, 2008) and CSCs display EMT properties (Brabletz *et al*, 2005; Mani *et al*, 2007). Most cancer types consist of heterogeneous cell populations (Fidler and Hart, 1982; Heppner and Miller, 1983; Nowell, 1986) and accumulating evidence reveals that heterogeneous cancer cells are not equivalent of tumour evolution; instead, CSCs are the components of evolution selection – that is, driving cancer progression, metastasis and drug resistance (Bonnet and Dick, 1997; Reya *et al*, 2001; Visvader and Lindeman, 2008; Greaves, 2013).

Evidence supports prostate CSCs (PCSCs) being critical in PC metastasis (Kelly and Yin, 2008). Prostate cancer stem cell were reported to express $CD44^+\alpha_2\beta_1^{hi}CD133^+$ (Collins *et al*, 2005).

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Malignant PC cells ectopically expressing human telomerase reverse transcriptase displayed the $CD44^+\alpha_2\beta_1^{hi}CD133^+$ surface profile and generated heterogeneous xenograft tumours (Gu *et al*, 2007; Miki *et al*, 2007; Li *et al*, 2008). In addition, the $CD44^+$ subpopulation isolated from several cultured PC cell lines were more tumorigenic than its isogenic $CD44^-$ populations (Patrawala *et al*, 2006). Prostate stem cells (PSCs) have been identified in both humans and mouse (Leong *et al*, 2008; Goldstein *et al*, 2010). Lineage-tracking analysis in a mouse model of PC revealed cells with PSC markers producing PC (Choi *et al*, 2012; Wang *et al*, 2013). Akin to orchiectomy in mouse leads to robust prostate involution and androgen removal results in marked PC regression, both PSC and PCSCs are androgen-independent. Whereas PSCs regenerate the prostate, PCSCs produce recurrent HRPCs (Qin *et al*, 2012). The signatures of PCSCs are associated with PC bone metastasis and poor prognosis (Colombel *et al*, 2012; Wang *et al*, 2013).

One of the signature proteins of normal and CSCs is aldehyde dehydrogenase (ALDH) (Moreb *et al*, 2008; Douville *et al*, 2009). The human genome consists of 19 ALDHs, including six and four members in the ALDH1 and ALDH3 families, respectively (Vasiliou and Nebert, 2005; Marchitti *et al*, 2009). ALDHs have important roles in detoxification by oxidising a wide variety of aldehydes to their corresponding carboxylic acids (Vasiliou *et al*, 2000). ALDH activity, especially that derived from ALDH1A1 and ALDH3A1, is associated with a variety of stem cells and CSCs (Ma and Allan, 2011; Zhang *et al*, 2011; Gasparetto *et al*, 2012; Vasiliou *et al*, 2013). Elevation of ALDH1A1 was reported in the tumour-initiating cells of LNCaP, PC3, DU145 and 22Rv1 human PC lines (Li *et al*, 2010; Nishida *et al*, 2013; Sefah *et al*, 2013). In addition, ALDH1A1 staining was detected in primary PC (Le Magnen *et al*, 2013), although others reported weak or absence of ALDH1A1 staining (Li *et al*, 2010; van den Hoogen *et al*, 2010). Instead, ALDH7A1 was observed in primary and bone-metastasised PC (van den Hoogen *et al*, 2010, 2011).

To further investigate the association of ALDH with PC, we have investigated the expression of ALDH3A1 in a set of PC cell lines, representing immortalised prostate epithelial cells, androgen-dependent and -resistant PC lines, non- and stem cells of PC, and demonstrated an association of ALDH3A1 with the progression of PC. In our examination of xenograft tumors of local and lung metastasis and xenograft tumors produced by either non-stem PC cells or PCSCs, elevation of ALDH3A1 is clearly associated with the level of severity of PC. This observation is also supported by our evaluation of ALDH3A1 expression in prostate glands, PIN lesions, PC, as well as local, lymph node and bone metastatic prostate carcinoma. Collectively, we provide evidence that ALDH3A1 expression correlates with PC progression.

MATERIALS AND METHODS

Tissue culture. LNCaP, PC3 and DU145 were purchased from American Type Culture Collection (ATCC) and cultured in RPMI-1640 (LNCaP), F12 (PC3) and MEM (DU145) supplemented with 10% FBS (Sigma Aldrich, Oakville, ON, Canada) and 1% Penicillin–Streptomycin (Life Technologies, Carlsbad, CA, USA). Immortalised human prostate epithelial BPH-1 cells were kindly provided by Dr Simon Hayward at the Vanderbilt University Medical Center, Nashville, TN, USA. BPH-1 cells are SV40 large T antigen-immortalised human prostate epithelial cells (Hayward *et al*, 1995). The tumorigenic and androgen-independent LNCaP derivative, C4-2, was kindly provided by Dr Martin Gleave at The University of British Columbia, Vancouver, BC, Canada (Thomas *et al*, 2011). LNCaP C4-2 cells were derived from LNCaP tumours maintained in castrated mice, and thus are castration-resistant with increased metastatic potential (Thalmann *et al*, 1994).

Generation of DU145 spheres. DU145 PC stem-like cells (spheres) were isolated and propagated as we have previously published (Rybak *et al*, 2011). In brief, DU145 monolayer cells were individualised with TrypLE Express solution (Life Technologies) and subsequently resuspended at a density of 5000 cells ml^{-1} in serum-free (SF) media (DMEM/F12 at a 3 : 1 mixture; Life Technologies) containing 0.4% bovine serum albumin (Bioshop Canada Inc., Burlington, ON, Canada) supplemented with 0.2 × concentration of B27 minus Vitamin A (Life Technologies) in T75 flasks. Typical spheres were formed in 10–12 days.

Collecting primary PC and metastatic PC. Prostate tissues were collected from patients who underwent prostate biopsies or radical prostatectomy at St. Joseph's Hospital in Hamilton, ON, Canada under the approval from the local Research Ethics Board (REB no. 11-3472) and with consent from patients.

Gene expression analysis. Total RNA was isolated from DU145 and DU145 PC stem-like cells with TRIZOL (Life Technologies, Burlington, ON, Canada). Gene expression was examined using the Affymetrix Human Gene 1.0 ST microarrays, which was purchased through the University Health Network Microarray Center (UHNMAC, www.microarrays.ca, Toronto, ON, USA). Procedures were carried out at UHNMAC according to the protocol detailed by Affymetrix.

Western blot analysis. Cell lysates were prepared in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 $\mu g\,ml^{-1}$ leupeptin and 10 $\mu g\,ml^{-1}$ aprotinin (Sigma Aldrich). A total of 50 μg of cell lysates were separated on SDS–PAGE gel and transferred onto Amersham hybond ECL nitrocellulose membranes (Amersham, Baie d'Urfe, QC, Canada). The membranes were blocked with 5% skimmed milk and then incubated with the indicated antibodies at 4 °C overnight. Appropriate HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. Signals were detected using an ECL Western Blotting Kit (Amersham). The primary and secondary antibodies and the concentrations used were as follows: mouse anti-ALDH1A1 (44/ALDH, 1 : 2000, BD Biosciences, Mississauga, ON, Canada), rabbit anti-ALDH3A1 (B-8, 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-actin (C-11, 1 : 1000, Santa Cruz Biotechnology), donkey anti-goat (SC2033, 1 : 3000, Santa Cruz Biotechnology), sheep anti-mouse (NA931, 1 : 3000, GE Healthcare, Mississauga, ON, Canada) and donkey anti-rabbit (NA943, 1 : 3000, GE Healthcare).

Immunohistochemistry (IHC). Prostate cancer tissue slides and normal human small intestines were deparaffinised in xylene, cleared in ethanol series and heat-treated (excluding slides stained with ALDH1A1) for 30 min in sodium citrate buffer (pH = 6.0) in a food steamer. Primary antibodies specific for mouse anti-ALDH1A1 (44/ALDH, 1 : 400, BD Biosciences) and rabbit anti-ALDH3A1 (ab87604, 1 : 5000, Abcam, Cambridge, MA, USA) were incubated with the sections overnight at 4 °C. Negative controls were incubated with a nonspecific mouse, goat or rabbit IgG. Biotinylated secondary IgG, and Vector ABC reagent (Vector Laboratories, Burlingam, CA, USA) was subsequently added according to the manufacturer's instructions. Washes were performed with PBS. Chromogen reaction was carried out with diaminobenzidine (Vector Laboratories) and the sections were counterstained with haematoxylin (Sigma Aldrich). Normal human small intestines (US Biomax, Rockville, MD, USA) was used as a positive control and stained alongside all tissues stained

with ALDH1A1. A total of 3–10 images of each slides were taken depending on the size of the tissue with a light microscope (Olympus, Tokyo, Japan) and analysed using the ImagesScope software (Aperio, Vista, CA, USA). Scores obtained using the ImageScope software were converted to an H-Score using the formula [H-Score = % positive × (intensity + 1)] (Mehta *et al*, 2001; Randall *et al*, 2009). The H-Scores were subtracted from the H-Scores obtained from stroma regions that were used for background subtraction. Any value less than 0 because of nonspecific staining in the stroma that were otherwise absent from epithelial regions was counted as 0. Scores were assigned to a scale of 0–3 (0 – negative or background staining, 1 – weak staining, 2 – modest staining, 3 – strong staining).

Real-time PCR analysis. Total RNA was isolated using TRIZOL (Life Technologies). Reverse transcription was carried out using superscript III (Life Technologies) according to the manufacturer's instruction. In brief, 2 μg of RNA was converted to cDNA at 65 °C for 6 min followed by 2-min incubation on ice, 25 °C for 11 min, 50 °C for 60 min and 70 °C for 15 min. Real-time PCR primers used for all 19 human ALDH isoforms and actin are listed in Supplementary Table 1. Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Burlington, ON, Canada) in the presence of SYBR-green according to the manufacturer's instructions (Applied Biosystems). In brief, each reaction consisted of 1 μl cDNA, 0.25 μl forward primer (10 μM), 0.25 μl reverse primer (10 μM), 4.75 μl H_2O and 6.25 μl of SYBR-green master mix. The PCR reaction was carried out in

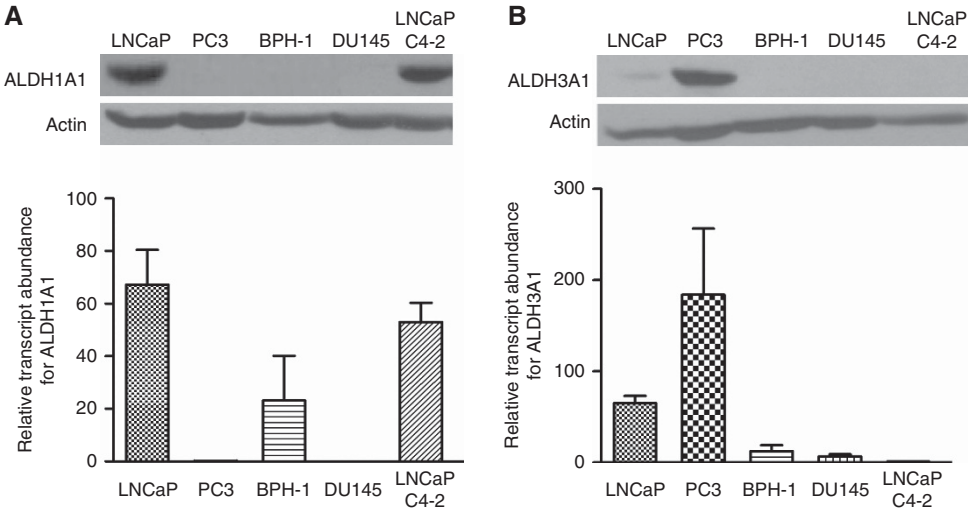

Figure 1. Expression of ALDH1A1 and ALDH3A1 in prostate epithelial and carcinoma cells. (**A**) Cell lysates and RNA were collected and isolated from the indicated cell lines and examined with western blot analysis for protein expression of ALDH1A1 (top) and with real-time PCR for mRNA levels (bottom). (**B**) Expression of ALDH3A1 was also examined for protein (top) and mRNA expression (bottom). β-actin was used as an internal control. Relative transcript abundance was determined according to a published procedure (van den Hoogen *et al*, 2010).

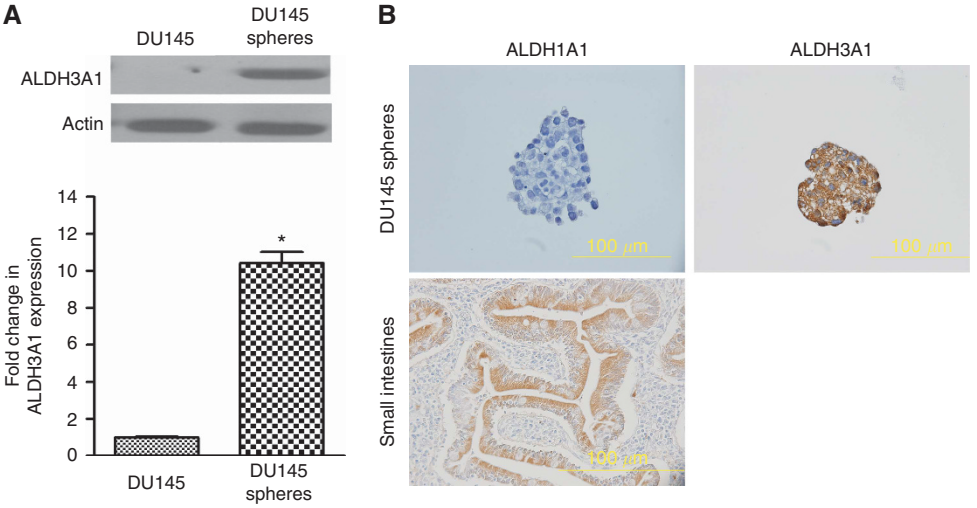

Figure 2. Upregulation of ALDH3A1 in DU145 prostate cancer stem cells. Prostate cancer-enriched stem-like cells were isolated as spheres from DU145. (**A**) Cell lysates were collected and western blot analysis was carried out for DU145 and DU145 spheres to determine ALDH3A1 expression (top). RNA was isolated from both cell lines and examined for ALDH3A1 mRNA expression using real-time PCR (bottom). β-actin was used as an internal control. ALDH3A1 mRNA in DU145 spheres is shown as a fold change to DU145 cells (mean ± s.d.). *$P<0.05$ by a Mann–Whitney test. (**B**) DU145 spheres were embedded in paraffin, sectioned and stained with immunohistochemistry for ALDH3A1 and ALDH1A1. Small intestine was used as a positive control.

a 96-well plate at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All samples were run in triplicate.

Formation of xenograft tumours and lung metastasis. A total of 10^6 DU145 monolayer (non-stem cells) and 10^4 sphere (stem-like) cells were individualised and resuspended in MEM/Matrigel mixture (1 : 1 volume), followed by implantation of 0.1 ml of this mixture subcutaneously into flanks of 8-week-old male NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME, USA). Mice were inspected for tumour appearance, by observation and palpation, and tumour growth was measured weekly using a caliper. Tumour volumes were determined using the standard formula: $L \times W^2 \times 0.52$, where L and W are the longest and shortest diameters, respectively. For the generation of lung metastasis, 10^6 individualised DU145 monolayer cells were resuspended into 0.3 ml of PBS and injected through the lateral tail vein of NOD/SCID mice. Mice were killed at 16 weeks post injection and their lungs were harvested and examined for tumour nodules. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

Statistical analysis. Statistical analysis was performed using Student's *t*-test or the Mann–Whitney test, and $P<0.05$ was considered statistically significant

RESULTS

Expression of ALDH3A1 in advanced PC cells. Among the 19 ALDH isoforms, ALDH1A1 is the most commonly reported ALDH to associate with CSCs and cancer progression, followed by ALDH3A1 (Marchitti *et al*, 2009; Ma and Allan, 2011), although ALDH1A3 and ALDH7A1 have recently been demonstrated to be associated with the metastasis of breast and PC, respectively (van den Hoogen *et al*, 2010; Marcato *et al*, 2011; van den Hoogen *et al*, 2011). Despite ALDH1A1 being examined in PC cell lines, including LNCaP, PC3, DU145 and 22Rv1 human PC lines (Li *et al*, 2010; Nishida *et al*, 2013; Sefah *et al*, 2013), the expression of ALDH3A1 has not yet been examined in PC cell lines. To determine ALDH3A1 expression during prostate tumorigenesis, we have examined both ALDH1A1 and ALDH3A1 in a panel of

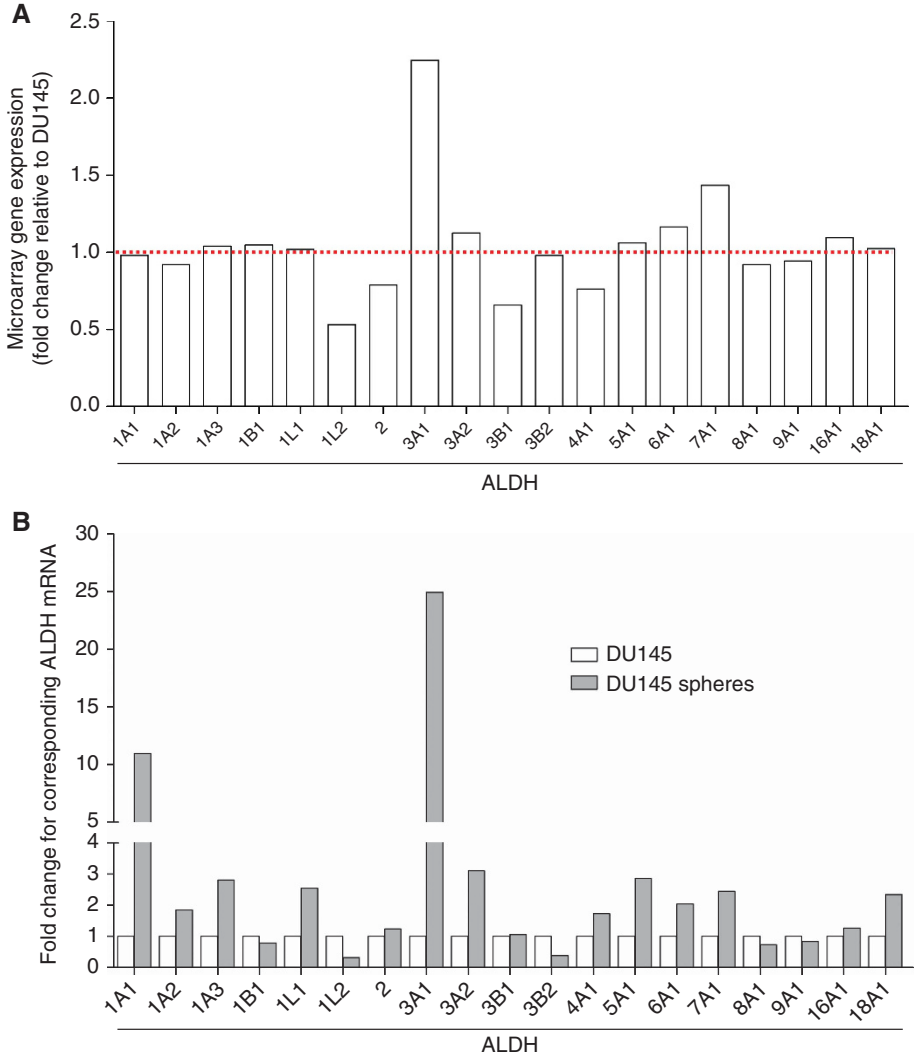

Figure 3. Specific increase in ALDH3A1 expression compared with DU145 in prostate cancer stem cells. (**A**) RNA was isolated from DU145 prostate cancer stem cells and DU145 cells and a microarray were carried out by the University Health Network Microarray Facility. Fold change was calculated as a ratio of the signal intensity of DU145 prostate cancer stem cells to DU145. A fold change of 1 (red line) denotes that both cell lines have relatively the same signal intensity. (**B**) Real-time PCR was carried out on all 19 ALDH genes for DU145 and DU145 prostate cancer stem cells. β-actin was used as an internal control. Corresponding ALDH mRNA in DU145 prostate cancer stem cells are shown as a fold change to DU145 cells (mean ± s.d.).

cell lines that reflect the process of prostate tumorigenesis, including immortalised but not transformed BPH-1 human prostate epithelial cells, androgen-dependent LNCaP, androgen-independent derivative of LNCaP C4-2, as well as androgen-independent DU145 and PC3 PC cells. Although both ALDH1A1 and ALDH3A1 were undetectable in BPH-1 cells (Figure 1), expression of either ALDH isoforms could be demonstrated at least in one PC cell line (Figure 1), supporting their common association with tumorigenesis (Marchitti *et al*, 2009; Ma and Allan, 2011). Consistent with the detection of ALDH1A1 in LNCaP cells (Qin *et al*, 2012), ALDH1A1 was present in LNCaP cells in both western and real-time PCR analyses but could not be detected by either methods for PC3 and DU145 cells (Figure 1A). We further examined LNCaP C4-2, a more aggressive subline derived from LNCaP cells, and detected ALDH1A1 expression in both western and real-time PCR analyses. On the other hand, ALDH3A1 was readily detected in androgen-resistant PC3 cells with a much lower level present in androgen-dependent LNCaP cells but not in LNCaP C4-2 cells (Figure 1B), supporting a substantially lower level of ALHD3A1 in LNCaP-based cell lines. The expression of ALDH1A1 and ALDH3A1 proteins correlates with their mRNA levels in BPH-1, LNCaP, LNCaP C4-2, PC3 and DU145 cells (Figure 1). Collectively, these observations indicate the association of ALDH3A1 with PC progression.

To take advantage of DU145 cells being able to produce stem-like cells (for simplicity, referred to as PC stem cells) in the form of spheres under defined SF conditions (Rybak *et al*, 2011), we postulated the possibility of whether ALDH1A1 and ALDH3A1 were undetectable in DU145 cells could be attributed to their specific association with DU145 PCSCs. Indeed in comparison with DU145 monolayer cells, ALDH3A1 was robustly upregulated in DU145 sphere (PCSCs) cells (Figure 2A), whereas ALDH1A1 could not be demonstrated using western blot when LNCaP cell lysate was used as a positive control (data not shown). In an IHC setting, ALDH3A1 but not ALDH1A1 was detected in DU145 sphere cells (Figure 2B). In addition, the ALDH1A1 mRNA level was very low and could only be detected using real-time PCR at cycle 32 (Supplementary Table 2) by using the published primers (van den Hoogen *et al*, 2010; Marcato *et al*, 2011). Taken together, the above results support the notion that ALDH3A1 expression associates with PC progression.

Specific increase in ALDH3A1 in DU145 PCSCs. To further consolidate the association of ALDH3A1 in DU145 PCSCs, we performed a microarray analysis of gene expression between DU145 monolayer and sphere cells (the PCSC population). Among all the 19 human ALDH isoforms, only ALDH3A1 was upregulated more than twofold (Figure 3A). These results were subsequently confirmed using real-time PCR analysis (Figure 3B). Although a high fold change is seen in ALDH1A1 for DU145 spheres, the mRNA expression for both DU145 and DU145 spheres was low. Once again, the mRNA levels were undetectable until cycle 32 for both cell lines (Supplementary Table 2). Collectively, these observations reveal that at least for DU145 cell-derived stem-like cells ALDH3A1 is increased in PCSCs.

Increases in ALDH3A1 in advance xenograft tumours derived from DU145 cells. We have previously demonstrated that xenograft tumours derived from DU145 stem-like cells displayed more characteristics that are associated with advanced PC compared with xenograft tumours generated from non-stem-like DU145 cells (Rybak *et al*, 2011). In particular, as $CD44^+$ DU145 cells were reported to be more tumorigenic than $CD44^-$ cells (Patrawala *et al*, 2006), we demonstrated the xenografts derived from DU145 stem-like cells consisted of more $CD44^+$ cells ($85.7 \pm 1.6\%$) compared with DU145 non-stem-like cells ($54.2 \pm 2.5\%$, $P<0.05$; Rybak *et al*, 2011). To take advantage of this knowledge, we produced xenograft tumours from DU145 monolayer and sphere cells. Whereas ALDH1A1 could not be detected in xenograft tumours produced by either cell lines, ALDH3A1 was detected in the xenograft tumours derived from both lines (Figure 4). In comparison with the low level of ALDH3A1 present in DU145 monolayer cell-derived xenograft tumours (see Discussion for details), higher levels of ALDH3A1 was apparent in DU145 PCSC-produced xenograft tumours (Figure 4), a common observation obtained in all three xenograft tumours produced from either DU145 monolayer or sphere cells (data not shown).

To consolidate the association of ALDH3A1 with advanced xenograft tumours, we generated lung metastasised tumours by tail vein injections of DU145 cells in NOD/SCID mice (Figure 5A). Significant elevation of ALDH3A1 in lung metastasised tumours were clearly demonstrated in comparison with xenograft tumours produced by subcutaneous implantation of DU145 cells (Figure 5B). Collectively, the above observations reveal the elevation of ALDH3A1 following increasing severity of PC.

Association of ALDH3A1 with PC progression. Abnormalities in prostate epithelial cells result in the precancerous PIN lesions, which progress to invasive carcinomas with increasing grade and

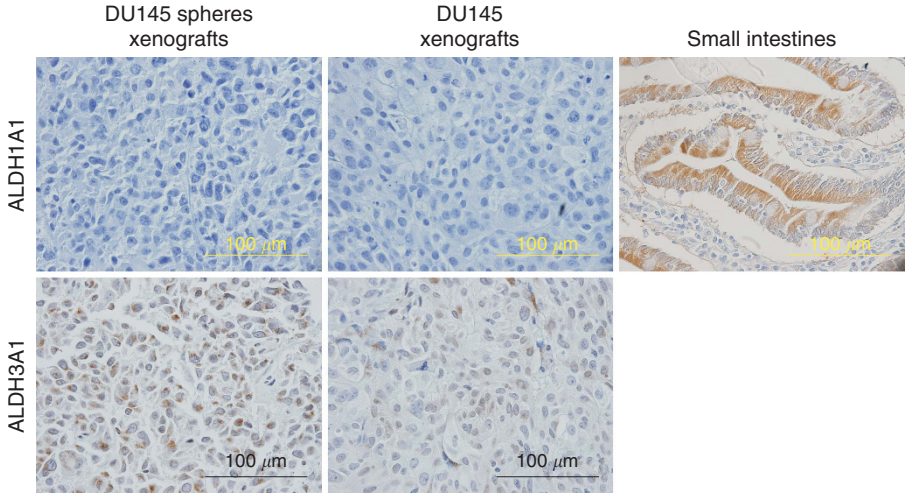

Figure 4. Expression of ALDH1A1 and ALDH3A1 in prostate cancer xenograft tumours. Xenograft tumours derived from DU145 ($n=3$) and DU145 spheres ($n=3$) through subcutaneous injections into immunocompromised mice were stained for ALDH1A1 and ALDH3A1 using immunohistochemistry. Human small intestines were used as a positive control for ALDH1A1.

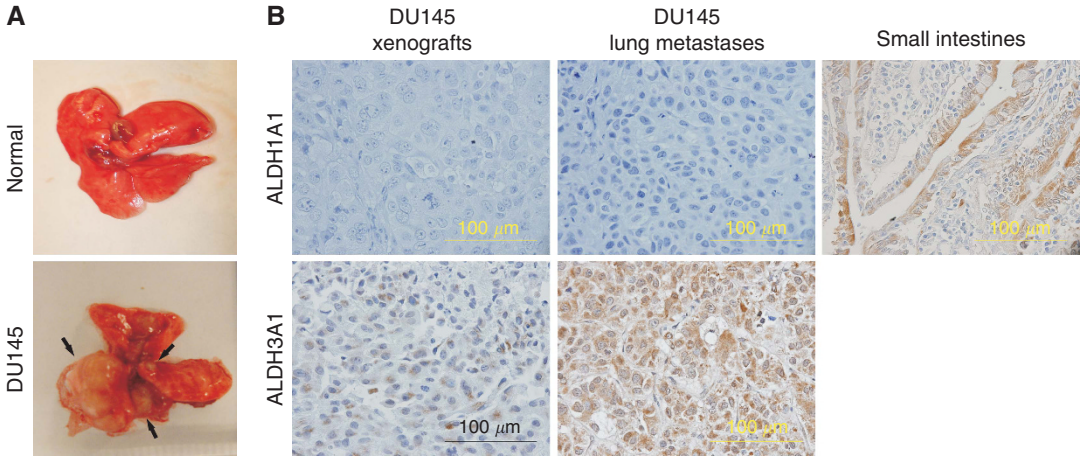

Figure 5. Expression of ALDH1A1 and ALDH3A1 in metastatic prostate cancer xenograft tumours. (**A**) A total of 0.3 ml of 10^6 DU145 cells in PBS was injected intravenously through the tail vein of immunocompromised mice. Lungs were harvested at 16 weeks post injection and tumours can be seen in lungs injected with DU145 cells (black arrows). (**B**) Lung tissues with metastatic nodules were embedded in paraffin, sectioned and stained for ALDH1A1 and ALDH3A1 ($n = 2$).

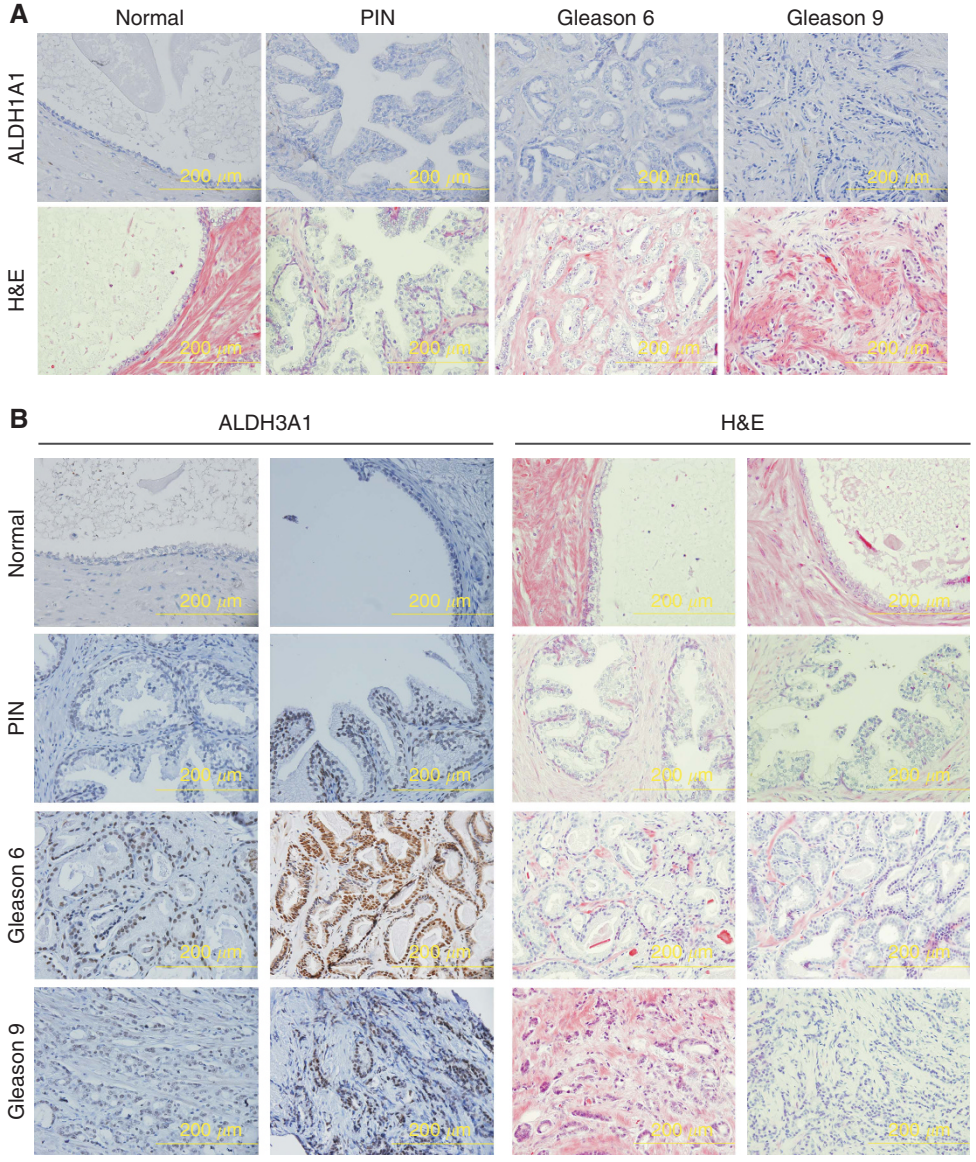

Figure 6. ALDH3A1 but not ALDH1A1 is expressed in primary prostate cancer tissues. (**A**) Primary prostate cancer tissues were stained for ALDH1A1 ($n = 6$) and (**B**) ALDH3A1 ($n = 47$). Typical images are shown for normal, PIN, Gleason 6 and 9 carcinomas.

stage. Local carcinomas subsequently progress to lymph node and bone metastasis (Ross, 2007; Moon *et al*, 2008). Consistent with this knowledge, we have examined ALDH1A1 and ALDH3A1 expression in a set of primary prostate tumours of different severity and metastatic prostate tumours (Supplementary Table 3). We were able to show that neither ALDH1A1 nor ALDH3A1 was detected in prostate glands (Figure 6); ALDH3A1 but not ALDH1A1 was present in PIN lesions, albeit low levels and in carcinomas (Figure 6). Despite the variations in ALDH3A1 staining in PIN lesions and in carcinomas, significantly higher levels of ALDH3A1 were observed in cancer compared with PIN lesions and cancer compared with normal prostate glands (Figure 7A). In addition, the percentage of patients positive for ALDH3A1 staining increases from PIN lesions to carcinoma (Figure 7B). Interestingly, the trend can also be noted within a number of patients with absent staining in normal, followed by the progression of weak to strong staining in PIN lesions and carcinoma, respectively (Figure 7C). Correlation with ALDH3A1 and Gleason score could not be determined, which might be attributed to the limited patient populations studied; however, a positive trend can be seen from patients expressing ALDH3A1 from low Gleason to high Gleason carcinomas (Figure 7D).

We subsequently examined ALDH1A1 and ALDH3A1 in metastatic PC. In three pairs of local and lymph node metastatic prostate tumours examined, ALDH1A1 was absent in all three cases and higher levels of ALDH3A1 was observed in metastatic PC compared with the local carcinomas (Figure 8A and B). As bone is the most frequent site for PC metastasis, we also examined four bone metastatic prostate tumours. Consistent with lymph node metastases, high levels of ALDH3A1 were observed in all four bone metastatic tumours (Figure 8C) and was absent for ALDH1A1 (data not shown). Taken together, we provide evidence that ALDH3A1 levels associate with increasing severity of PC.

DISCUSSION

It has been well established that high levels of ALDH activity ($ALDH^{hi}$) coexist with cancer cells, observations that are in accordance with ALDH's role in detoxification. Among the 19 ALDH isoforms, ALDH1A1 is the most widely studied and possesses both metabolic and nonmetabolic functions. Ubiquitously expressed in many tissues, ALDH1A1 is best known for its conversion of retinal to retinoic acid (RA), in addition to its protective function in the eye and ability to oxidise highly reactive aldehydes (Marchitti *et al*, 2009; Chen *et al*, 2012; Vasiliou *et al*, 2013). Overexpression of ALDH1A1 in lung cancer cells and leukemia cells increased proliferation and drug resistance (Moreb *et al*, 2012). Whereas breast cancer patients treated with cyclophosphamide revealed high levels of ALDH1A1 in metastatic tumour cells that survived exposure to cyclophosphamide and in metastatic tumours that did not respond to cyclophosphamide treatment (Sládek *et al*, 2002), demonstrating a role of ALDH1A1 in developing chemoresistance.

In agreement with CSCs being critical for developing chemoresistance and cancer evolution, cancer cells possessing $ALDH^{hi}$ display elevated tumorigenicity capacity, indicative of the association of $ALDH^{hi}$ with CSCs (Moreb *et al*, 2008; Douville *et al*, 2009). High ALDH activity was first identified as a characteristic feature of haematopoietic stem cells (Kastan *et al*, 1990; Jones *et al*, 1995) and has a critical role in regulating haematopoietic stem cell self-renewal and differentiation (Chen *et al*, 2012). High levels of

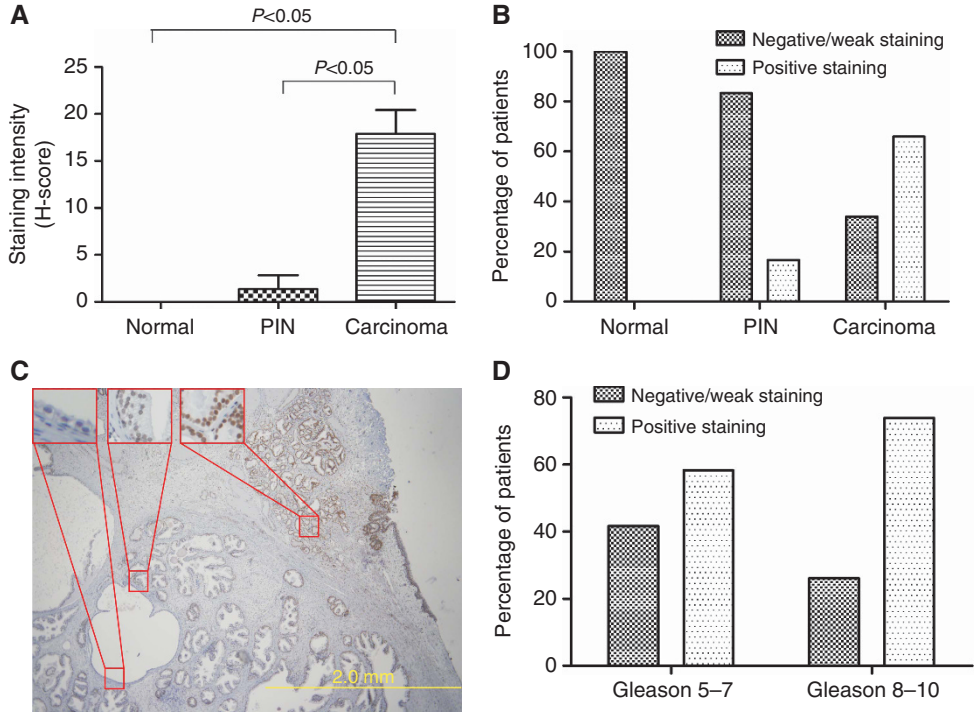

Figure 7. ALDH3A1 is associated with prostate cancer progression. (**A**) A total of 47 primary prostate cancer tissues were IHC-stained for ALDH3A1 (normal $n=6$, PIN $n=6$, Gleason 5–7 $n=24$, Gleason 8–10 $n=23$). The H-score was calculated for each patient and grouped into normal, PIN or carcinoma. Staining intensity according to the H-score was graphed (mean ± s.d.). Note: the H-score for normal was close to 0. (**B**) Percentage of patients examined expressing negative to weak or positive ALDH3A1 staining was calculated. (**C**) Primary prostate cancer tissue was stained for ALDH3Al. Regions negative for staining in normal glands, weak staining in PIN and strong staining in carcinoma can be observed. (**D**) The percentage of patients expressing negative to weak or positive staining with low Gleason or high Gleason prostate cancer was examined and calculated.

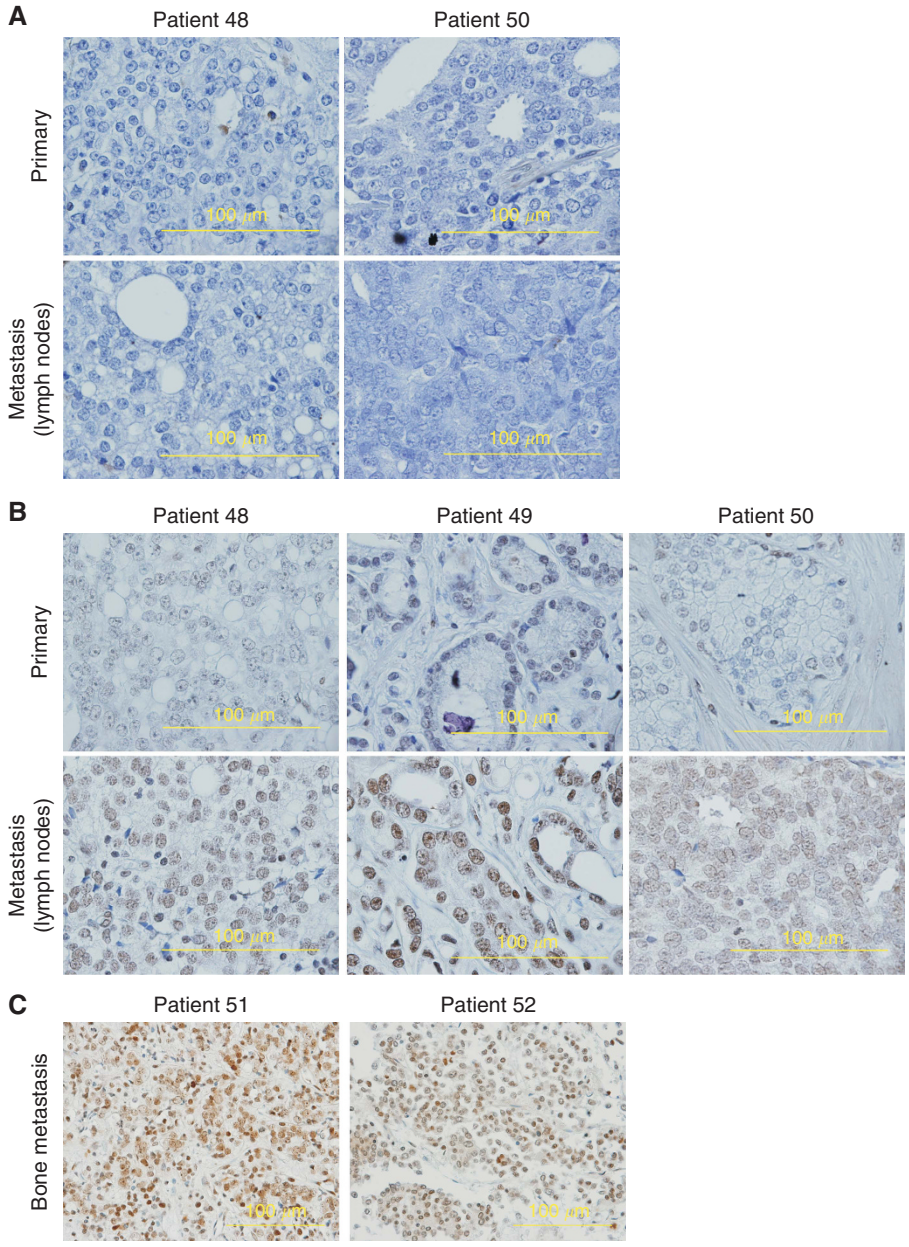

Figure 8. ALDH3A1 is expressed in metastatic prostate cancer. (**A**) Three primary prostate cancer patients with lymph node metastasis were examined for ALDH1A1. (**B**) Expression for ALDH3A1 was also examined in the three primary prostate cancer tissue and their corresponding lymph node metastatic prostate cancer. (**C**) Four prostate cancer bone metastatic tumours were stained and examined for ALDH3A1 expression. Representative images for two patient samples are shown.

ALDH is associated with poor prognosis in breast, lung and ovarian carcinomas (Ginestier *et al*, 2007; Jiang *et al*, 2009; Charafe-Jauffret *et al*, 2010; Deng *et al*, 2010; Landen *et al*, 2010). The same situation has also been repeatedly demonstrated in PC (Burger *et al*, 2009; Li *et al*, 2010; van den Hoogen *et al*, 2010; Doherty *et al*, 2011; Hellsten *et al*, 2011; Germann *et al*, 2012; Nishida *et al*, 2012; Qin *et al*, 2012; Nishida *et al*, 2013). Cellular ALDH activity is commonly detected using a fluorescence-based Aldefluor assay. Although this assay has been regarded to be specific for ALDH1A1, recent studies demonstrated that ALDH activity detected with Aldefluor in CSCs is attributed to individual ALDH isoforms (van den Hoogen *et al*, 2010; Chen *et al*, 2011; Marcato *et al*, 2011; Vasiliou *et al*, 2013) – that is, the assay is not ALDH1A1-specific. This is consistent with ALDH isoforms having a broad range of substrate specificity (Koppaka *et al*, 2012). Although an increase in ALDH1A1 in Aldefluor^{high} cells could be demonstrated in cultured PC cell lines (Li *et al*, 2010; Germann *et al*, 2012; Nishida *et al*, 2012, 2013), whether the Aldefluor^{high} cells were derived from ALDH1A1 remains unclear. However, only recently has there been evidence demonstrating the presence of ALDH1A1 in PC (Le Magnen *et al*, 2013). In addition, ALDH7A1 but not ALDH1A1 was also detected in primary PC types (van den Hoogen *et al*, 2010). This is consistent with our inability to detect ALDH1A1 in DU145 cell-produced xenograft tumours, lung metastasis and primary prostate cancer tissues.

We show here the expression of ALDH3A1 in xenograft tumours and primary PC tissues, with the levels of ALDH3A1 correlating with increasing severity of PC. This concept is supported by the observed higher levels of ALDH3A1 in androgen-refractory PC3 compared with androgen-dependent LNCaP cells as well as the elevation of ALDH3A1 in DU145-derived PCSCs in comparison with the non-stem cell population.

Whereas ALDH3A1 could not be demonstrated in DU145 monolayer cells (Figure 1), low levels of the protein were detected in the xenograft tumours produced by DU145 monolayer cells (Figure 4). This suggests that a certain level of dedifferentiation may have occurred; this dedifferentiation may have produced PCSCs, which then contributed to tumorigenesis. This possibility is in line with the higher levels of ALDH3A1 present in DU145 PCSC-produced xenograft tumours. Intriguingly, lung metastases derived from ALDH3A1-negative DU145 monolayer cells produced substantially elevated ALDH3A1 expression (Figure 6B), indicating the importance of ALDH3A1 in PC tumorigenesis. Collectively, these observations support the theme that elevation of ALDH3A1 associates with the progression of PC.

As a member of the ALDH family, ALDH3A1 possesses multiple functions including its importance in maintaining haematopoietic stem cells, functioning as a corneal crystallin with protective functions in the eye for UV radiation, regulating cell proliferation and cell cycle and conferring resistance to chemotherapeutic agents (Canuto *et al*, 1999; Wang *et al*, 2001; Muzio *et al*, 2003; Pappa *et al*, 2005; Muzio *et al*, 2006; Moreb *et al*, 2007; Gasparetto *et al*, 2012; Singh *et al*, 2013; Vasiliou *et al*, 2013). All the above functions may be relevant to PC evolution; this scenario fits well with the robust upregulation of ALDH3A1 in DU145 cell-derived spheres and PCSCs are likely the source of PC evolution. In supporting this possibility, ALDH1A1 has a role in metastatic breast cancer resistant to chemotherapy treatment (Sládek *et al*, 2002). As ALDH3A1 is expressed in PC, it would be interesting to examine its contributions to chemoresistance.

Whereas we observed the association of ALDH3A1 with PCSCs *in vitro*, its expression in advanced PC (lung (Figure 5B), lymph node and bone metastases (Figure 8B and C)) is rather uniform. It is possible that ALDH3A1-positive population contains PCSCs – that is, ALDH3A1 is not a marker of PCSCs *in vivo*. Alternatively, ALDH3A1-positive PC cells may possess plasticity to dedifferentiate to PCSCs. Evidence in favour of this possibility is the generation of ALDH3A1-positive cells in xenograft tumours that were produced from ALDH3A1-negative cells (Figure 4). Regardless of what might be the possibility, our study demonstrated that the association of ALDH3A1 with PC progression agrees with PCSCs mediating PC evolution.

As the association of ALDH1A1 and ALDH7A1 with PC progression has recently been clearly demonstrated (van den Hoogen *et al*, 2010, 2011; Le Magnen *et al*, 2013), it is tempting to suggest that high levels of ALDH3A1, ALDH1A1 and ALDH7A1 are associated with prostate tumorigenesis; the detailed contributions of either to PC many require further investigations. The ALDH1 isoform is most commonly associated with human cancer types, including the carcinomas of lung, liver, pancreas, ovaries, cervix, breast, colon and bladder (Ginestier *et al*, 2007; Huang *et al*, 2009; Ucar *et al*, 2009; Lingala *et al*, 2010; Sun and Wang, 2010; Ma and Allan, 2011). However, further investigation is required to examine the role of ALDH1A1 in PC progression. Intriguingly, ALDH1A1, 1A2 and 1A3 are well known for the oxidation of retinal (vitamin A aldehyde) to RA (Koppaka *et al*, 2012), and RA induces cell differentiation via binding to RA receptors (Gupta *et al*, 2012). In accordance with this knowledge, decrease in ALDH1A2 in PC was associated with poor prognosis (Kim *et al*, 2005), and the reduction of both ALDH1A1 and ALDH1A2 was reported in the prostate of TRAMP (transgenic adenocarcinoma mouse prostate) mice in comparison with age-matched non-transgenic mice (Touma *et al*, 2009).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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