

**Full Title:**

**WNT SIGNALLING REGULATES SELF-RENEWAL AND DIFFERENTIATION OF PROSTATE CANCER CELLS WITH STEM CELL CHARACTERISTICS**

**Running Title**

WNT regulates prostate cancer stem cell attributes

**Key words**

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**ABSTRACT**

Prostate cancer cells with stem cell characteristics were identified in human prostate cancer cell lines by their ability to form from single cells self-renewing prostaspheres in non-adherent cultures. Prostaspheres exhibited heterogeneous expression of proliferation, differentiation and stem cell associated markers CD44, ABCG2 and CD133. Treatment with WNT inhibitors reduced both prostasphere size and self-renewal. In contrast, addition of Wnt3a caused increased prostasphere size and self-renewal, which was associated with a significant increase in nuclear  $\beta$ -catenin, keratin 18, CD133 and CD44 expression. As a high proportion of LNCaP and C4-2B cancer cells express androgen receptor we determined the affect of the androgen receptor antagonist bicalutamide. Androgen receptor inhibition reduced prostasphere size and expression of PSA, but did not inhibit prostasphere formation. These effects are consistent with the androgen independent self renewal of cells with stem cell characteristics and the androgen dependent proliferation of transit amplifying cells. As the canonical WNT signalling effector  $\beta$ -catenin can also associate with the androgen receptor. We propose a model for tumour propagation involving a balance between WNT and androgen receptor activity. That would affect the self-renewal of a cancer cell with stem cell characteristics and drive transit amplifying cell proliferation and differentiation. In conclusion, we provide evidence that WNT activity regulates the self-renewal of prostate cancer cells with stem cell characteristics independently of androgen receptor activity. The inhibition of WNT signalling therefore has the potential to reduce the self-renewal of prostate cancer cells with stem cell characteristics and improve therapeutic outcome.

## INTRODUCTION

Prostate cancer is a leading cause of male cancer related deaths <sup>[1]</sup>. In those who are not suitable for attempts at cure with radiotherapy or surgery, treatment is by androgen deprivation therapy, which effectively shrinks androgen-dependent tumours. However, this usually leads to recurrent androgen-independent prostate cancer, which undergoes metastasis <sup>[2, 3]</sup> with a median survival time of 20-24 months <sup>[4]</sup>. The majority of human prostate adenocarcinoma cells express luminal cell markers that include androgen receptor (AR), prostate specific antigen (PSA), keratins 8 and 18 <sup>[5]</sup>. This has led to speculation that prostate cancers are derived from either mature luminal cells that have acquired proliferation ability, transit-amplifying epithelial cells that precede luminal cell differentiation or from androgen-independent prostate stem cells <sup>[6, 7]</sup>. A role for stem cells is suggested by the regenerative capacity of rodent prostate glands following androgen ablation <sup>[8]</sup>, the ability of some prostate cancer cells to survive androgen deprivation therapy and to subsequently progress to metastatic androgen independent prostate cancer <sup>[5, 6]</sup>, and the finding that AR negative human prostate cancer cells with stem cell characteristics can give rise to AR positive cells <sup>[9, 10]</sup>.

Cancer cells that are capable of self-renewal and differentiation, properties of normal tissue stem cells, have been shown to be the fundamental cause of both human acute myeloid and lymphoid leukemia <sup>[11, 12]</sup>. These cells have been termed “cancer stem cells” and there is increasing evidence that cells with stem cell characteristics are also the driving force behind tumour formation in many solid tissue cancers including brain, colon and breast <sup>[13-15]</sup>. Non-adherent, defined media culture techniques have been used to isolate neuronal, colon and breast cancer cells with stem cell characteristics <sup>[15-18]</sup>. The use of this culture system has recently been extended to cells derived from the prostate and it has been reported that hTERT immortalised primary prostate cancer cells can form spheres which were termed “prostaspheres” <sup>[19]</sup> and that dissociated primary murine prostate stem cells demonstrate clonal

self renewal in prostate sphere cultures <sup>[20]</sup>. In this study we report the use of non-adherent sphere cultures for clonal growth of single cells from human prostate cancer cell lines.

As the majority of androgen independent cancer cells express the AR <sup>[21]</sup>, the aberrant activation of the AR in low circulating levels of androgen may contribute to the growth of androgen independent prostate cancers <sup>[5]</sup>. Several proteins have been described which could potentiate AR transactivity and contribute to androgen independent prostate cancer <sup>[5]</sup>. One such protein is  $\beta$ -catenin <sup>[22]</sup>, the major effector of canonical WNT signalling <sup>[23]</sup>. WNT signalling can be inhibited by Dickkopf-1 (DKK1), which directly binds to the cell surface protein LRP5/6 (associated with the Frizzled family of WNT receptors) and blocks canonical WNT signaling associated with nuclear  $\beta$ -catenin <sup>[24-26]</sup>. Or by Soluble Frizzled Related Proteins (sFRP), which resemble the cysteine rich ligand-binding domain of the Frizzled family and are believed to function as extracellular WNT inhibitors <sup>[27, 28]</sup>. Mutations that stabilize  $\beta$ -catenin occur in a variety of human tumours and can lead to accumulation of nuclear  $\beta$ -catenin <sup>[23]</sup>. In prostate cancer activation by mutation is reported in 5% of tumours <sup>[29, 30]</sup>. However, WNT signalling may also play a significant role in the development and progression of metastatic androgen independent prostate cancer, as 25-38% of these cancers have tumour cells with nuclear  $\beta$ -catenin localization <sup>[29, 30]</sup>. Increased nuclear  $\beta$ -catenin accumulation in prostate cancer cells could cause transcriptional changes through interaction with TCF family transcription factors or the AR. In this study, we show that WNT signalling can regulate the self renewal of prostate cancer cells with stem cell characteristics independently of AR activity.



## RESULTS

### **A stem cell-like subpopulation exists within prostate cell lines**

Utilising the technique of non-adherent suspension culture or sphere formation (Figure 1a) we have identified a subpopulation of cells with prostasphere forming ability within the prostate cancer cell lines 22RV1, DU145, PC3, VCaP, LNCaP and the LNCaP subline C4-2B.

Whereas the majority of cells in suspension culture failed to proliferate, rare cells (0.1-8%) displayed the ability to grow as spherical prostaspheres (Figure 1b). The efficiency of prostasphere formation (P0) varied and was highest in the cell types LNCaP and its androgen independent derivative C4-2B, which express AR and PSA <sup>[31]</sup>.

### **Self-renewal, proliferation and characterisation of prostaspheres**

To confirm that the LNCaP and C4-2B prostaspheres were the progeny of an individual cell, rather than the aggregation of quiescent cells, we performed clonal growth assays with single cells derived from P0 prostaspheres (Figure 1c). Following P0 prostasphere dissociation and plating at 1 cell per well, we observed the generation of 1-3 new spheres (P1-P2) from single cells (Figure 1d). These prostaspheres were typically 25-100µm in diameter after 6 days. At 12 days only 20% of LNCaP prostaspheres were greater than 100µm in diameter, compared to 60% of C4-2B prostaspheres. The 12 day LNCaP spheres were the same size as C4-2B spheres cultured for 6 days (Figure 1e). It was also possible to generate further new spheres from single dissociated cells of P1-2 prostaspheres. This clonal protasphere self-renewal was observed for 4 passages with LNCaP cells and 8 passages with C4-2B cells (Figure 1d). To estimate the number of cells contained in an individual prostate sphere and the total number of cells that could be derived from a single P0 cell, we used the method described by Reynolds and Rietze <sup>[32]</sup>. C4-2B derived spheres after 12 days in culture had a median diameter of 100µm and an average of 1,000 cloned cells. As the cells derived from spheres have been

diluted to only 1 cell/well (Figure 1c) at each passage this suggests that a self-renewing C4-2B P0 cell has the capacity to generate, after eight passages, 128,000 daughter cells in 128 spheres, (based on our observation that each sphere on average gave rise to two daughter spheres and would contain 1000 cells/100 $\mu$ m sphere). As the number of LNCaP spheres reduced with serial passage and the LNCaP spheres had a reduced median size, this suggests the self-renewing LNCaP P0 cell had the capacity to generate 2,250 daughter cells in 5 spheres (containing 450 cells/75 $\mu$ m sphere).

### **Prostasphere characterisation**

Keratin 5/14 expression was not detected in adherent cultures, rarely detected in LNCaP spheres and significantly expressed in C4-2B spheres ( $p < 0.0001$ ; Figure 2). In contrast, keratin 18 expression was detected in both adherent cultures (LNCaP 37%; C4-2B 40%) and prostaspheres with LNCaP cells expressing higher levels than C4-2B cells ( $p < 0.014$ ; Figure 2). Nuclear AR expression was detectable in both LNCaP and C4-2B adherent cultures and prostaspheres, with the majority of both adherent and prostasphere LNCaP and C4-2B cells expressing AR; however negative cells could also be observed (Figure 2). No significant differences were observed between the levels of AR expression in prostasphere cultures (Figure 2). The expression of nuclear Ki67 (indicating cells in the active phases of the cell cycle: G1, S, G2 and mitosis) was significantly higher in the C4-2B than the LNCaP spheres ( $p < 0.0001$ ; Figure 2). CD133, ABCG2 and CD44 were enriched in both LNCaP and C4-2B prostaspheres compared to adherent cultures (Figure 3). In both LNCaP and C4-2B adherent cultures we were able to identify rare CD133 positive cells (0.05-0.4% of the total population) following MACS CD133 enrichment (Figure 3j). CD133 and ABCG2 expression was detected in 40% of LNCaP spheres, whereas 100% of C4-2B spheres were positive for these markers. The number of cells expressing CD133 was significantly higher in C4-2B spheres

compared to LNCaP spheres (Figure 3m). C4-2B spheres on average contained 2 CD133 positive cells compared to 0.4 CD133 positive cells per LNCaP sphere ( $p=0.014$ ) (Figure 3m). Interestingly, nuclear AR expression was not detected in CD133 positive cells (Figure 2i). The expression of ABCG2 was also significantly higher in C4-2B spheres, which on average contained 10 ABCG2 positive cells compared to 1 ABCG2 positive cell per LNCaP sphere ( $p=0.0001$ ; Figure 3m). Similar levels of CD44 expression was detected in both cell types (1-3 positive cells per sphere;  $p=0.74$ ; Figure. 3m).

### **Androgen receptor activity affects prostasphere size**

As AR activity could contribute to cell proliferation and sphere self-renewal in both LNCaP and C4-2B cultures. We determined the affects of AR inhibition using the AR antagonist bicalutamide, which inhibits receptor activity in both LNCaP and C4-2B cells. Bicalutamide treatment was associated with a decrease in sphere size in both cell types after 12 days in culture but an increase in the ability of P0 dissociated cells to generate P1 spheres (Figure 4a,b). Consistent with bicalutamide inhibiting AR activity we observed a significant decrease in the expression by prostasphere cells of the androgen responsive protein PSA (LNCaP,  $p=0.003$ ; C4-2B,  $p=0.014$ ) and the AR (LNCaP,  $p=0.014$ ; C4-2B,  $p=0.002$ ; Figure 4c). A reduction in expression of Ki67 following bicalutamide treatment was only observed in C4-2B cells ( $p=0.001$ ; Figure 4c). Whereas, CD133 expression was not significantly affected in either cell type by bicalutamide treatment (Figure 4d).

### **WNT signalling regulates prostasphere formation and self-renewal**

As WNT dysregulation is associated with a variety of cancers and WNT activity can regulate stem cells in a number of tissues we evaluated the affects of WNT signalling on prostasphere formation. Wnt3a or two secreted inhibitors of WNT signalling were added to dissociated prostate cells derived from LNCaP or C4-2B P0 spheres (Figure 5). The Wnt3a treated cells

generated larger prostaspheres (Figure 5a,b,c,d,g), with two-fold more Wnt3a C4-2B spheres obtaining a diameter of more than 100 $\mu$ m. In addition, the ability to form spheres was significantly increased by Wnt3a treatment of dissociated P1 LNCaP and P1 LNCaP C4-2B cells (Figure 5h). Wnt3a treated C4-2B P2 and P3 spheres also generated a similar 1.5 fold increase in sphere formation. In contrast, DKK1 and SFFRP2 treatment was associated with a decrease in C4-2B prostasphere size (no spheres were observed which were >100 $\mu$ m in diameter) and formation (Figure 5e,f, g, h). WNT inhibitor treatment blocked significantly ( $p=0.04$ ) the self-renewal ability of P0 C4-2B prostaspheres, with 60% fewer secondary spheres forming in the treated groups. No significant differences in the viability or levels of apoptosis of cells within spheres following Wnt3a or DKK1 treatment were observed (Figure 5i-m). Using trypan blue dye exclusion staining, 98.7% cells were viable in DKK1 treated spheres compared to 100% untreated spheres.

### **Characterization of Wnt3a treated prostaspheres**

The C4-2B prostaspheres treated with Wnt3a were analysed for expression of prostate, cell cycle and stem cell associated markers (Figure 6). Keratin 18 expression was significantly increased in the Wnt3a spheres (C4-2B control 16% K18<sup>+</sup>; C4-2B+Wnt3a 61% K18<sup>+</sup>,  $p<0.005$ ,  $n=5$ ; Figure 6a,d,m). The majority of the cells in the spheres were positive for Ki67 and although the expression of Ki67 was higher in the Wnt3a treated spheres (87% of cells Ki67 positive) in comparison to untreated cells (74% of cells Ki67 positive) the difference was not statistically significant ( $p=0.066$ ,  $n=5$ ). The majority of cells were AR positive (87-94%) and no significant difference in nuclear AR expression was observed following Wnt3a treatment (Figure 6c,f,m). We then determined expression of CD133, ABCG2 and CD44 in both control and Wnt3a treated prostaspheres. CD133 and CD44 positive cells were both significantly higher in Wnt3a treated spheres (Figure 6g,j,h,k,n). Control C4-2B spheres on

average contained 2 CD133 positive cells whereas in Wnt3a treated spheres 5 CD133 positive cells were typically detected ( $p=0.04$ ,  $n=8$ ). CD44 expression was found in 5 cells per C4-2B sphere whereas 11 positive cells were found on average in a Wnt3a treated sphere ( $p=0.045$ ,  $n=5$ ). Similar levels of ABCG2 expression was detected in untreated and Wnt3a spheres (10-11 positive cells per sphere;  $p=0.73$ ,  $n=5$ ). The analysis of CD133 and CD44 expression by flow cytometry confirmed the presence of rare CD133Hi (1%) and CD44Hi (1.4%) cells in the prostaspheres. The addition of Wnt3a increased the CD133Hi population 2.2 fold without affecting the CD44Hi (1.5%) population. Wnt3a treated prostaspheres were dissociated and sorted for CD133Hi or CD44Hi cells and cultured for 10 days to determine sphere forming ability. CD44Hi cells failed to form spheres whereas 2-3% of CD44Lo cells formed spheres. In contrast, 2-3% of both CD133Hi and CD133Lo selected cells formed spheres. CD44Hi cells therefore have a reduced ability to form spheres while both CD133Hi and CD133Lo cells can form spheres.

Nuclear  $\beta$ -catenin expression was also affected by Wnt3a treatment.  $\beta$ -catenin was strongly detected in untreated spheres at cell junctions and only rarely found in the nucleus, whereas Wnt3a treatment was associated with increased nuclear  $\beta$ -catenin accumulation (Figure 6o,p). The nuclear (blue channel corresponding to DAPI) and  $\beta$ -catenin (red channel) pixel intensity was quantified on a defined nuclear section (figure 6r,s)<sup>[33]</sup>. The mean nuclear  $\beta$ -catenin fluorescence value was significantly higher in Wnt3a treated cells (fluorescence value of 120) compared to control cells (fluorescence value of 64;  $p<0.0001$ ), with significantly more Wnt3a treated cells expressed high levels of nuclear  $\beta$ -catenin (28%) compared to control cells (4%;  $p<0.005$ ) (Figure 6q).

### **The WNT effects are independent of androgen receptor activity**

We next investigated the interaction between WNT and AR signalling. We treated dissociated C4-2B cells for 6 days with Wnt3a (C4-2B+Wnt3a), bicalutamide (C4-2B+Bic) and bicalutamide plus Wnt3a (C4-2B+Bic+Wnt3a). Wnt3a treatment on its own caused an increase in sphere size, whereas bicalutamide was associated with a decrease in sphere size, as expected. The diameter of spheres co-treated with Wnt3a and bicalutamide was similar to the group treated with Wnt3a alone. CD133 expression increased following Wnt3a but not bicalutamide treatment (Figure 7). This Wnt3a induced increase in CD133 expression was not inhibited by bicalutamide treatment (Figure 7). As expected bicalutamide decreased the number of prostasphere cells expressing the AR (78% untreated cells, 54% bicalutamide treated cells,  $p=0.036$ ; Figure 7). Interestingly, the addition of Wnt3a to bicalutamide treated cells was associated with a further reduction in AR expression (41%), demonstrating that Wnt3a does not induce AR expression.

## DISCUSSION

The cancer stem cell hypothesis proposes that tumour cells *in vivo* are organised as a hierarchy with a cell with stem cell characteristics at the apex, which has the ability to self-renew and generate differentiated progeny (Figure 9) <sup>[34]</sup>. Normal murine prostate stem cells have been functionally identified by their ability to form prostate spheres <sup>[20]</sup> and to form differentiated prostate tubular structures when returned to an *in vivo* environment <sup>[20, 35]</sup>. The *in vivo* generation of prostate structures from normal human prostate cells in xenograft studies and the ability to isolate a human basal prostate cell population with enriched capacity for prolonged clonal expansion and luminal differentiation ability has led to the hypothesis that normal human prostate stem cells are located within the basal layer of the gland <sup>[36-39]</sup>. Several candidate markers of prostate stem cells have been proposed including  $\alpha 2\beta 1$  integrin, Sca-1, CD44, CD133, CD117 and the ATP-binding cassette transporter ABCG2, which is also associated with the resistance of cancer cells to chemotherapeutic agents <sup>[35, 38, 40, 41]</sup>. However, many of these markers are reported to be expressed in both stem and non-stem cells, including stromal and differentiated cells <sup>[35, 42, 43]</sup>. Although, CD117 has recently been reported to be a marker of murine prostate stem cells and a single  $\text{Lin}^- \text{CD117}^+ \text{CD44}^+ \text{CD133}^+ \text{Sca-1}^+$  murine prostate cell has been shown to generate prostate epithelial tubules when combined with rat stromal cells under the renal capsule <sup>[35]</sup>. Whether CD117 also defines a human prostate stem cell has not been demonstrated.

There is increasing evidence that human prostate cancers are also hierarchal with rare tumour initiating cells that are distinct from the non-tumour initiating, bulk population of the tumour <sup>[10]</sup>. This has led to the proposal that prostate cancer cells with stem cell characteristics may contribute to tumour initiation and progression <sup>[44, 45]</sup>. Prostate cancer cells demonstrating clonogenic growth and heterogeneous progeny have been enriched from human prostate tissue by selection with  $\alpha 2\beta 1$  integrin or CD133 <sup>[10]</sup>. In addition, carcinoma derived cell lines from

prostate (DU145 and PC3) exhibit adherent *in vitro* colony patterns<sup>[46, 47]</sup>, that are similar to the tightly adherent holoclones of primary keratinocytes in adherent clonogenicity assays<sup>[48]</sup> and are consistent with the retention of self-renewing cells with stem cell characteristics. Although, these colonies patterns are not generated by LNCaP cells which are only lightly attached to the substrate in adherent cultures, do not become confluent and tend to clump (Bisson and Prowse, unpublished observations). The distinctive PC3 holoclones but not the paraclones, were also tumourigenic in xenograft assays, (showing the presence of self-renewing tumour initiating cells) and enriched for CD44,  $\alpha 2\beta 1$  integrin and  $\beta$ -catenin<sup>[47]</sup>. CD44 is an adhesion molecule with multiple isoforms, which is expressed in both stem and differentiated cell populations and has pleiotropic roles in signalling, migration and homing<sup>[34]</sup>. Interestingly CD44<sup>HI</sup> LNCaP, LAPC and DU145 cells have also been reported to be enriched in tumour initiating cells<sup>[49, 50]</sup>. However, it should also be noted that 9 of 10 implants of 1000 LAPC9 CD44<sup>-</sup> $\alpha 2\beta 1$ <sup>+</sup> cells<sup>[49]</sup> and two of 5 implants of 100 DU145 CD44<sup>-</sup> cells<sup>[50]</sup> formed xenograft tumours in these studies. The xenograft assay system may also not support the growth of all cancer stem cell populations. As tumour cells engraftment and growth requires an intricate network of interactions with diverse supporting murine cells (including fibroblasts, endothelial cells, macrophages, mast cells, and mesenchymal stem cells), and many of the cytokines and receptors required for these two-way interactions are incompatible between mice and humans<sup>[51, 52]</sup>.

Non-adherent sphere cultures have also been used to functionally enrich and identify neural, breast and colon cancer cells with stem cell characteristics. In this study we have investigated the ability of prostate cancer cell lines to form prostaspheres in non-adherent cultures. We found that all the prostate cancer cell lines tested had the ability to form spheres. Although heterogeneity existed in the percentage of cells of each cancer lines that were able to form spheres, which could reflect differences in the frequency of cells with stem cell



characteristics or differences in adaption to non-adherent culture. Interestingly, cell lines that are both AR negative (PC-3, DU145) and positive (LNCaP, VCaP, 22RV1) were able to form prostaspheres. Primary cells from human prostate cancers were also found to form spheres (Bisson and Prowse, unpublished), which is in agreement with the finding that hTERT immortalised primary prostate cancer cells form prostaspheres<sup>[19, 53]</sup>. Prostasphere formation was most prominent in the androgen-dependent LNCaP cancer cell line and the androgen-independent LNCaP subline C4-2B, where up to 8% of single cells could form spheres. This is consistent with recent reports that cancer stem cells need not be rare<sup>[54]</sup>.

As well as their ability to self-renew from a single cell, the analysis of marker expression revealed that the prostaspheres contained a heterogeneous mixture of cells, expressing markers associated with both differentiation (keratin 18 and PSA) and proliferation (Ki67), consistent with prostaspheres originating from a cancer cell with stem cell characteristics. The smaller size and lower potential for self-renewal of LNCaP spheres compared to C4-2B spheres, would suggest that LNCaP cells with stem cell characteristics identified using the prostasphere assay, have a greater tendency to differentiate during prolonged culture under these conditions. This is in keeping with the lower expression of LNCaP prostasphere cells of the cell cycle marker Ki67, higher levels of keratin 18 and low levels of keratin 5 (which has been associated with a more aggressive prostate cancer cell phenotype)<sup>[55, 56]</sup>. The expression of the stem cell associated markers CD133 and CD44 was also significantly enhanced in non-adherent culture and highest in C4-2B prostasphere cells. However, while C4-2B CD133Hi cells formed prostaspheres, CD133 and CD44 selection by FACS failed to enrich for C4-2B cells with prostasphere forming ability. This may indicate phenotypic heterogeneity between cancer cell populations, as CD133 and CD44 do not always appear to tightly mark a cell population with stem cell characteristics<sup>[49, 57]</sup> and also reflects the challenge that definitive cancer stem cell markers have not been identified<sup>[34, 51]</sup>.

We also investigated the expression of AR and ABCG2 in the prostasphere cultures. As AR can regulate both the proliferation and differentiation of prostate cells [3, 5] and ABCG2 has been reported to identify stem cells [58-61] or a transit amplifying populations [49, 62] and is also linked with dihydrotestosterone efflux [63], which could confer reduced sensitivity to androgens. Interestingly, the expression of ABCG2 was significantly higher in C4-2B prostaspheres. Whereas AR was expressed by the majority but not all LNCaP and C4-2B prostasphere cells. We therefore determined whether AR activity contributed to cell proliferation and sphere self-renewal in both LNCaP and LNCaP C4-2B cultures, utilising the AR antagonist bicalutamide, which is widely used to treat patients with hormone naïve prostate cancer and is associated with a reduction in PSA levels [64]. Bicalutamide inhibits the activity and expression of AR in adherent LNCaP cultures [64, 65], causing an AR specific, progressive inhibition of cell growth and an associated reduction in the percentage of S-phase cells [66]. Bicalutamide also inhibits the proliferation of adherent cultures of C4-2B cells [67]. Bicalutamide treatment reduced the size of both LNCaP and LNCaP C4-2B prostaspheres but did not inhibit prostasphere formation. The bicalutamide treated spheres were associated with a significant decrease in both AR and PSA expression, consistent with the expected effects of bicalutamide treatment [64, 68]. No inhibition of CD133 levels were observed, suggesting AR activity does not contribute to CD133 expression. A significant reduction in the cell cycle marker Ki67 following bicalutamide treatment was only observed in C4-2B prostaspheres. This may be due to the presence of a larger population of C4-2B transit amplifying cells that are dependent on AR activity for proliferation. The inhibition of AR activity also caused a small but significant increase in the relative sphere number in both cell types, which is consistent with prostasphere formation being driven by a small population of C4-2B cells that can self-renew independently of AR activity.

WNT signalling has also been implicated in the regulation of stem cells in a number of tissues and its dysregulation is associated with a variety of cancers<sup>[23]</sup>. As overexpression of WNT growth factors<sup>[25, 69]</sup> and reduced expression of WNT inhibitors<sup>[25, 70]</sup> are reported in prostate cancer and WNT growth factors can also regulate adherent human prostate cancer cell growth<sup>[71, 72]</sup>. We investigated whether WNT signalling affects prostasphere formation using the small molecule inhibitors DKK1 (which blocks the canonical WNT pathway) and sFRP2 (which blocks both canonical and non-canonical WNT signalling)<sup>[28]</sup>. We have found that WNT pathway inhibition causes a significant decrease in prostasphere size and relative sphere formation independently of apoptosis. As predicted by the inhibitor studies, treatment with the ligand Wnt3a, which is not expressed by C4-2B prostate cancer cells and is an activator of canonical WNT signalling<sup>[25, 71]</sup>, caused a significant increase in prostasphere size and self-renewal. This was associated with a significant increase in the expression of nuclear  $\beta$ -catenin, in accordance with the ability of Wnt3a to activate canonical WNT signalling. This suggests that WNT treatment promotes the self-renewal of prostate cancer cells with stem cell characteristics. Consistent with reports that Wnt3a signalling can preserve an undifferentiated phenotype in CD133+ human cord blood-derived cells<sup>[73]</sup>, Wnt3a supports embryonic stem cell self-renewal<sup>[74]</sup>, WNT/ $\beta$ -catenin selectively enhanced mammosphere forming ability of Sca1+ murine mammary cells<sup>[75]</sup> and detection of high levels of  $\beta$ -catenin in PC3 holoclones. We also found that WNT treatment was associated with increased CD133 expression, which may be a direct effect as TCF/LEF1 binding sites are reported in the CD133 promoter<sup>[76]</sup>. However, although the CD133 positive cells were often AR negative, CD133 enrichment failed to enrich for prostasphere forming ability. The function of CD133 in LNCaP and C4-2B cells is therefore uncertain, although it has been suggested that CD133 functions as an organizer of plasma membrane topology<sup>[77]</sup>. Interestingly, Wnt3a treatment also caused increased expression of the differentiation associated keratin 18. This is consistent with WNT

signalling being able to promote not only stem cell renewal but also terminal differentiation in the gut crypt and hair follicle lineage<sup>[23, 78]</sup>. An association of WNT signalling with differentiation may therefore be a normal mechanism to promote homeostasis, by balancing self renewal and proliferation with the induction of differentiation, thus preventing uncontrolled cell expansion<sup>[23]</sup>.

Moreover, since firstly, the effect of AR inhibition suggests the self renewal of cells with stem cell characteristics is an androgen independent event and AR activity may cause these cells to become androgen dependent transit amplifying cells. Secondly, WNT signalling can also be affected by AR expression, as  $\beta$ -catenin can directly interact with the TCF transcription factors or the AR, causing reduced transcription of TCF responsive genes and enhanced AR activity<sup>[72]</sup>. This suggests a model for tumour propagation involving a cellular hierarchy where the balance between WNT and AR activity regulates both the self-renewal of a cancer cells with stem cell characteristics and the proliferation/differentiation of transit amplifying cells (Figure 8).

In conclusion, we provide evidence that WNT activity regulates the self-renewal of prostate cancer cells with stem cell characteristics independently of AR activity. This is consistent with the initial response of prostate cancers to hormone therapy and the subsequent progression to androgen independent prostate cancer<sup>[3, 79]</sup>. In addition, WNT activity may not only maintain prostate cancer it may also promote metastasis, by contributing to the mechanism through which prostate cancer cells with stem cell characteristics induce osteoblastic activity in the bone<sup>[25]</sup>. The inhibition of WNT signalling therefore has the potential to reduce the uncontrolled prostate cell self-renewal that drives cancer, moderate metastasis and improve therapeutic outcome.

## MATERIALS AND METHODS

### Cell culture and prostasphere formation

Prostate cancer cell lines PC3, VCAP, LNCaP, 22RV1 and DU145 were purchased from the European Collection of Cell Cultures through Sigma-Aldrich). The androgen independent LNCaP subline C4-2B was purchased from Viomed Laboratories. Androgens are essential for the optimal growth of LNCaP but not C4-2B cells. C4-2B cells were originally derived following growth of LNCaP cells transplanted into castrated athymic mice<sup>[80]</sup>. C4-2B cells proliferate as fast in the absence as the presence of androgens and over-express AR<sup>[3]</sup>. Both LNCaP and C4-2B cells express a common gain-of function AR mutant (that remains sensitive to the drug bicalutamide)<sup>[31]</sup>. All cells were cultured at 37°C in RPMI media without phenol red (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) or 10% (v/v) charcoal stripped FBS (Hyclone), 2.4mM glutamine (Sigma-Aldrich), 1% (v/v) pyruvate (Sigma-Aldrich), penicillin and streptomycin (50U and 50µg/ml) (Invitrogen). Cells were detached using trypsin (Sigma-Aldrich), then centrifuged and counted. On low attachment on 6 well plate (Costar), single cells were plated at 50cells/cm<sup>2</sup> in DMEM/F12 (Invitrogen ) supplemented with B27 and N2 (Invitrogen). Cells were grown under these conditions for 6-12 days and formed non-adherent P0 spheres, termed prostaspheres. The prostaspheres were dissociated with Accutase (Sigma-Aldrich) to generate single cells and re-plated into new low attachment plates. For the clonogenicity assay, the prostaspheres were diluted after 6 days in culture and transferred into 96 well low attachment plates (Costar) in order to isolate individual spheres. Each sphere was dissociated with Accutase (Sigma-Aldrich) at 37°C and regularly triturated by pipetting to generate single cells (20 minutes). By dilution one cell was plated per well of 96 low attachment plates containing DMEM/F12 with B27 and N2. Single cell cloning was confirmed by microscopic analysis.

**WNT inhibitors, Wnt3a and bicalutamide treatment**

Prostaspheres were dissociated with accutase (Sigma-Aldrich) to generate single cells and plated at 160 cells/well on 96 low attachment plate (Costar). The medium was supplemented with WNT inhibitors DKK1 at 4 $\mu$ g/ml (R&D Systems) or sFRP2 at 4 $\mu$ g/ml (BD Bioscience); Wnt3a at 5, 10 or 20ng/ml (R&D Systems) and the AR inhibitor bicalutamide at 1 $\mu$ M (Toronto Research Chemicals Inc) and prostaspheres counted and sized at 7 days. For self-renewal culture assays, individual untreated prostaspheres and those cultured with Wnt3a (20ng/ml) and/or bicalutamide (1 $\mu$ M) were serially dissociated with accutase and the cells replated into 3 wells (low attachment 96 well plates) and cultured for 6 days. Replicate independent experiments were performed.

**Tunel and Trypan Blue assays**

At least 200 spheres were passaged and cultured with and without Wnt3a at 20ng/ml. The spheres were dissociated using accutase and cells counted using trypan blue (Sigma-Aldrich) to calculate the percentage of live cells. The same number of spheres were used for the in-situ cell death assays (Tunel assay; Roche). Camptothecin (Sigma-Aldrich) treatment at 2.4 $\mu$ M, was used as a positive control for apoptosis. The spheres were incubated for 16 hours in chamber slides before staining, following the instruction of the manufacturer. The spheres were analysed by confocal microscopy (Zeiss LSM 510).

**Flow cytometry**

The prostaspheres after 5 to 7 days in culture were dissociated with accutase until single cells and labelled with anti CD133-PE (CD133/1 Myltenyi) or CD44-PE (clone) for 10 minutes in dark. The cells were sorted using the FACS Aria (BD biosciences). After sorting the cells were

seeded in 96 well plates in DMEM/F12 (Invitrogen ) supplemented with B27 and N2 (Invitrogen) and Wnt3a at 20ng/ml.

### **Immunofluorescence**

The prostaspheres were incubated for 16 hours in chamber slides before paraformaldehyde (4%) fixation at room temperature for 20 minutes<sup>[81]</sup>. The prostaspheres were washed and incubated with 0.1% triton (30 minutes), then washed and blocked with 0.2% BSA (15 minutes). Slides were incubated with the following antibodies: anti AR (dilution 1/50 rabbit IgG polyclonal PG-21, Upstate), ABCG2 (dilution 1/50 rat IgG2a monoclonal BXP-53, Abcam), keratin 18 (dilution 1/50 mouse IgG1 monoclonal DC10, Abcam), CD44 (dilution 1/50 mouse IgG2b monoclonal G44-26, BD Pharmingen), CD133 (dilution 1/100 rabbit polyclonal to CD133, Abcam), Ki67 (dilution 1/50 clone MIB-1, DakoCytomation) and  $\beta$ -catenin (dilution 1/500 mouse IgG1 clone 14/ $\beta$ -catenin, BD Transduction Laboratories). Slides were then washed with PBS and incubated (1 hour) with secondary antibodies labelled with Alexa 488 and 594 (Invitrogen). Control incubations with non-specific primary antibodies or secondary antibody alone did not label. Slides were finally washed with PBS and mounted using mounting solution containing DAPI before observation using a confocal microscope (Zeiss LSM 510).

### **Confocal $\beta$ -catenin analysis**

Prostaspheres images were analyzed for nuclear  $\beta$ -catenin expression following Wnt3a treatment. Confocal imaging was performed using a Zeiss LSM 510, 40x objectif zoom 2-3. Images were analyzed using personal computer-based LSM 510 and ImageJ software. To evaluate the level of  $\beta$ -catenin expressed in the cell nucleus, we selected distinct prostasphere

cells and defined nuclear horizontal sections corresponding to the DAPI nuclear staining (blue). We quantified the intensities of the pixels in each channel (DAPI: blue;  $\beta$ -catenin: red) on the sections and generated histograms. The levels of nuclear  $\beta$ -catenin were determined and mean values calculated within the nucleus <sup>[33]</sup>.

### **Statistical analysis**

Results of prostaspheres formation and sized were analyzed by *t*-test and/or chi-square test using Minitab software.  $p < 0.05$  was considered significant.



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### TITLES AND LEGENDS TO FIGURES

Figure 1. Prostasphere formation and culture. (a) Schematic representation of prostate cancer cell prostasphere self-renewal and proliferation. Adherent prostate cancer cells were dissociated and plated at low density in non-adherent cultures to form P0 spheres. Individual spheres were isolated, dissociated and serial dilution used to replate cells at 1 cell/well. After 6-10 days the new P1 spheres were counted, dissociated and a single cell/well replated (P2<sup>+</sup>), to demonstrate the ability of single cells to self-renewal. Dissociated spheres obtained in P0 were also serially plated in order to proliferate the spheres. (b) Prostasphere formation (P0) was observed with different prostate cell lines, with only a small percentage of cells being able to form spheres. (c) Typical LNCaP and C4-2B spheres generated from a single cell after 6 days and 12 days in non-adherent culture (scale bar 50µm). (d) LNCaP and C4-2B spheres formation relative to initial spheres counted at passage (P) 1 to 7. (e) Percentage of LNCaP and C4-2B spheres with diameters <100 µm, 100-150 µm or >150 µm.

Figure 2. Prostate cancer spheres derived from a single cell contain a heterogeneous population of proliferating and differentiating cells. Expression of proliferation and epithelial differentiation markers in LNCaP (a-c,g) and C4-2B (d-f,h) spheres after 12 and 7 days in culture respectively. Immunofluorescence showing K5/14 (a,d; green), K18 (b,e; in green), AR (c,f; red) and Ki67 (g,h; green). (i) Co-staining for AR (red) and CD133 (green) in a C4-2B sphere (arrow indicates lack of nuclear AR expression in a CD133 positive cell, enlarged in insert). Expression of proliferation and differentiation markers in adherent cultures of LNCaP (j-l) and C4-2B (m-o): K5/14 (j,m), K18 (k,n) and AR (l,o) where the arrows indicate AR negative cells. DAPI nuclear counterstaining (blue), scale bar 50 µm. (p) Percentage of

positive cells for Ki67, K15/14, K18 and AR expression per sphere. (Students t test:\* p=0.05-0.005; \*\* p<0.005).

Figure 3. Prostate cancer sphere cells express putative stem cell markers. LNCaP (a-c) and C4-2B (d-f) spheres after 12 and 7 days in culture, stained for CD133 (a,d), ABCG2 (b,e) and CD44 (c,f) immunofluorescence (green), DAPI nuclear counterstaining (blue), scale bar 50µm. Note the CD133 positive cells are indicated by the white arrows. Expression of CD133 (g,j), ABCG2 (h,k), CD44 (i,l) in LNCaP (g-i) and C4-2B (j-l) in adherent culture, scale bar 50µm. Insert (j) CD133 immunoreactivity in C4-2B cells cultured under adherent conditions following dissociation and MACS CD133 enrichment. (m) Number of CD133, ABCG2 and CD44 positive cells counted per LNCaP and C4-2B spheres. (Students t test:\* p=0.05-0.005; \*\* p<0.005).

Figure 4. AR activity is not required for sphere formation but affects sphere size. (a) Bicalutamide (Bic) treatment of LNCaP and C4-2B cells increased sphere formation after 12 days in culture. P0 proliferating LNCaP or C4-2B spheres were pooled at day 6, dissociated and 160 cells plated per well for each culture condition in triplicate. The relative number of spheres that formed after 12 days from 160 cells is shown. (b) Bicalutamide (Bic) treatment caused a reduction in sphere diameter in both LNCaP and C4-2B cells. Percentage of spheres categorized by diameter: < 50 µm, 50-100 µm, 100-150 µm and >150 µm, measured after 12 days in culture. (Students t test:\* p=0.05-0.005). (c) Bicalutamide treatment inhibits AR activity affecting PSA, AR expression and Ki67 expression. Percentage of PSA, AR and Ki67 positive cells in control spheres and spheres treated with bicalutamide. (d) Bicalutamide treatment does not affect the number of CD133 positive cells in control spheres and spheres treated with bicalutamide. (Students t test:\* p=0.05-0.005; \*\* p<0.005).



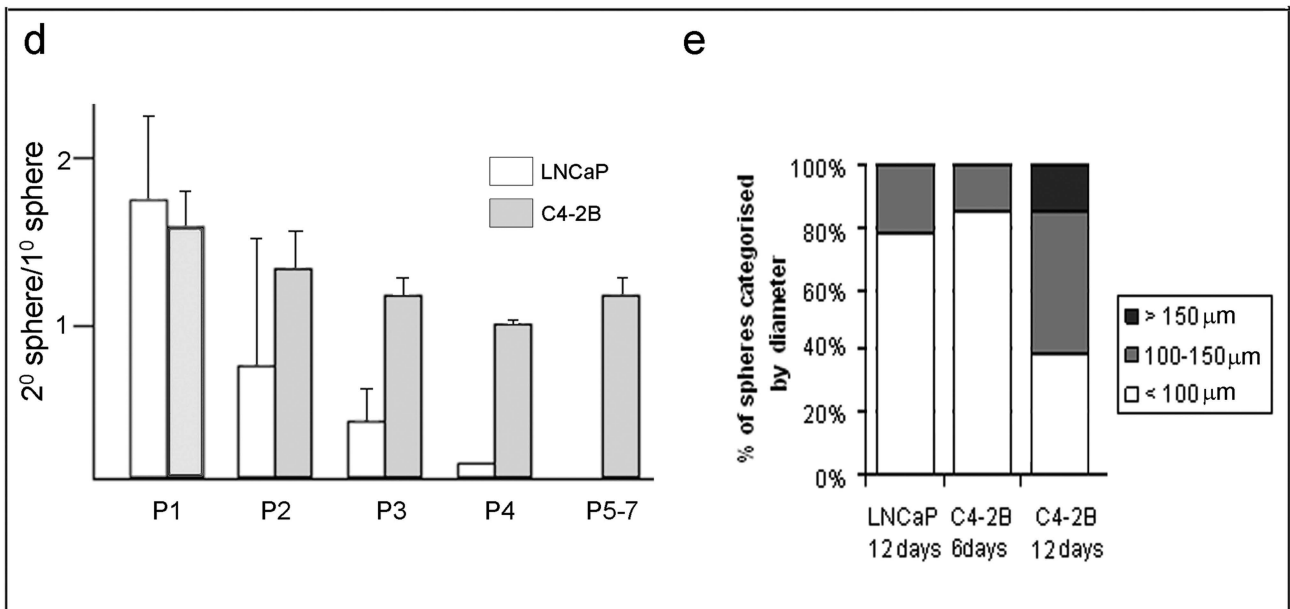
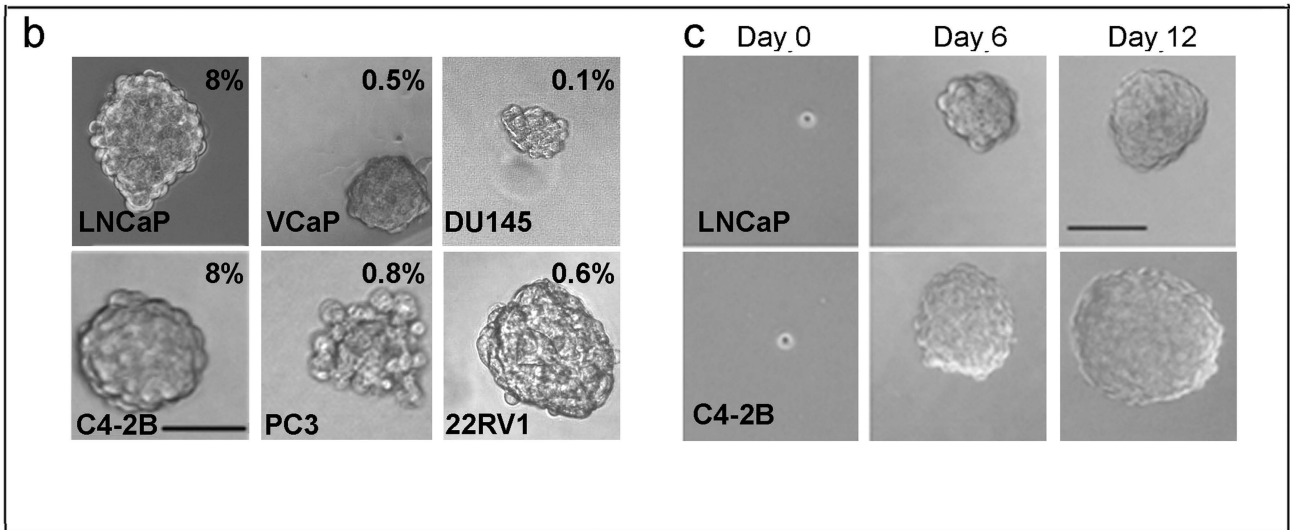
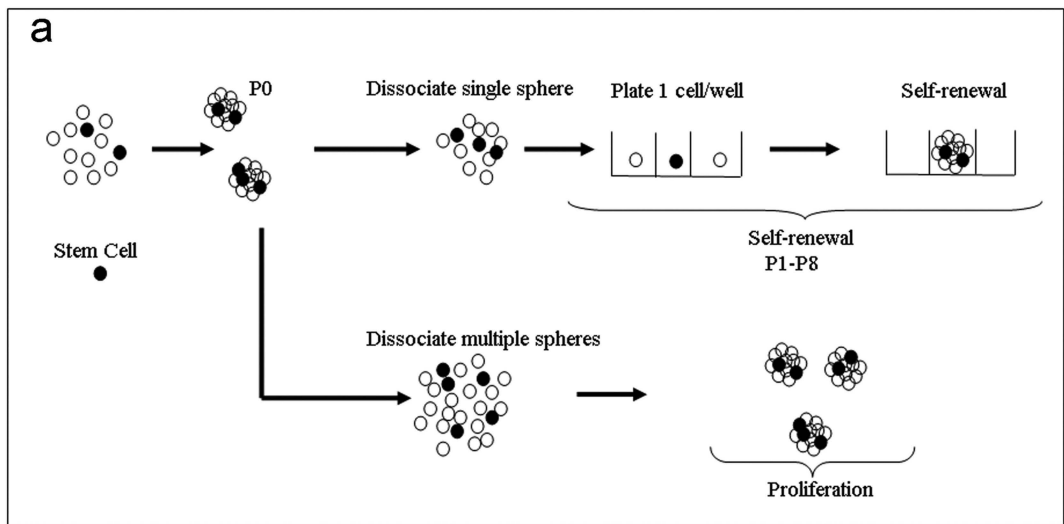
Figure 5. WNT activity regulates prostasphere formation. Representative LNCaP (a,b) and C4-2B (c,d) spheres generated from single cells after 12 days in non-adherent culture. Control cultures (a,c), Wnt3a (b,d), DKK1 (e) and sFRP2 (f). Scale bar 50 $\mu$ m. (g) Prostasphere size is increased by Wnt3a treatment and reduced by WNT inhibitors. (h) Prostasphere formation is increased by Wnt3a treatment and reduced by WNT inhibitors. (i-m) TUNEL in situ showing apoptotic cells are rare in control (i) C4-2B prostaspheres (5%) and not induced by Wnt3a (k) or DKK1 (l), whereas camptothecin treatment (j) induces apoptosis in 57% of cells (m). The results are the mean and standard deviation from 3 replicates (\* $p < 0.05$ ).

Figure 6. Wnt3a affects keratin 18, CD133, CD44 and nuclear localization of  $\beta$ -catenin. (a-n) Expression of markers in C4-2B control (a-c,g-i) and Wnt3a (d-f,j-l) treated spheres after 7 days in culture. Immunofluorescence showing K18 (a,d; green) Ki67 (b,e; green), AR (c,f; red), DAPI nuclear counterstaining (blue), scale bar 50  $\mu$ m. (m) Percentage of positive cells for K18, Ki67 and AR expression per sphere. The results are the mean and standard deviation of 4 replicates (\* $p < 0.05$ ). Immunofluorescence showing CD133 (g,j; green), CD44 (h,k; green) and ABCG2 (l; green) by immunofluorescence, DAPI nuclear counterstaining (blue), scale bar 50 $\mu$ m. (n) Number of positive cells for CD133, CD44 and ABCG2 counted per sphere. The results are the mean and standard deviation of 5 (CD44, ABCG2) and 8 (CD133) replicates (\* $p < 0.05$ ). (o,p) Confocal microscope images of  $\beta$ -catenin expression (Red) in control (o) and Wnt3a (p) treated spheres cultured for 7 days (white scale bar 10 $\mu$ m). The nucleus was stained by DAPI (blue). Nuclear  $\beta$ -catenin expression (red/pink) increased following Wnt3a treatment (o-q). The pixel intensities in each channel were quantified on the nucleus predefined confocal Z sections (yellow bar inserts). Typical numerical values of pixel intensities are shown plotted on histogram for  $\beta$ -catenin (red) and DAPI (blue) for a cell from (r) control and (s) Wnt3a treated spheres. A significant increase in mean fluorescence nuclear

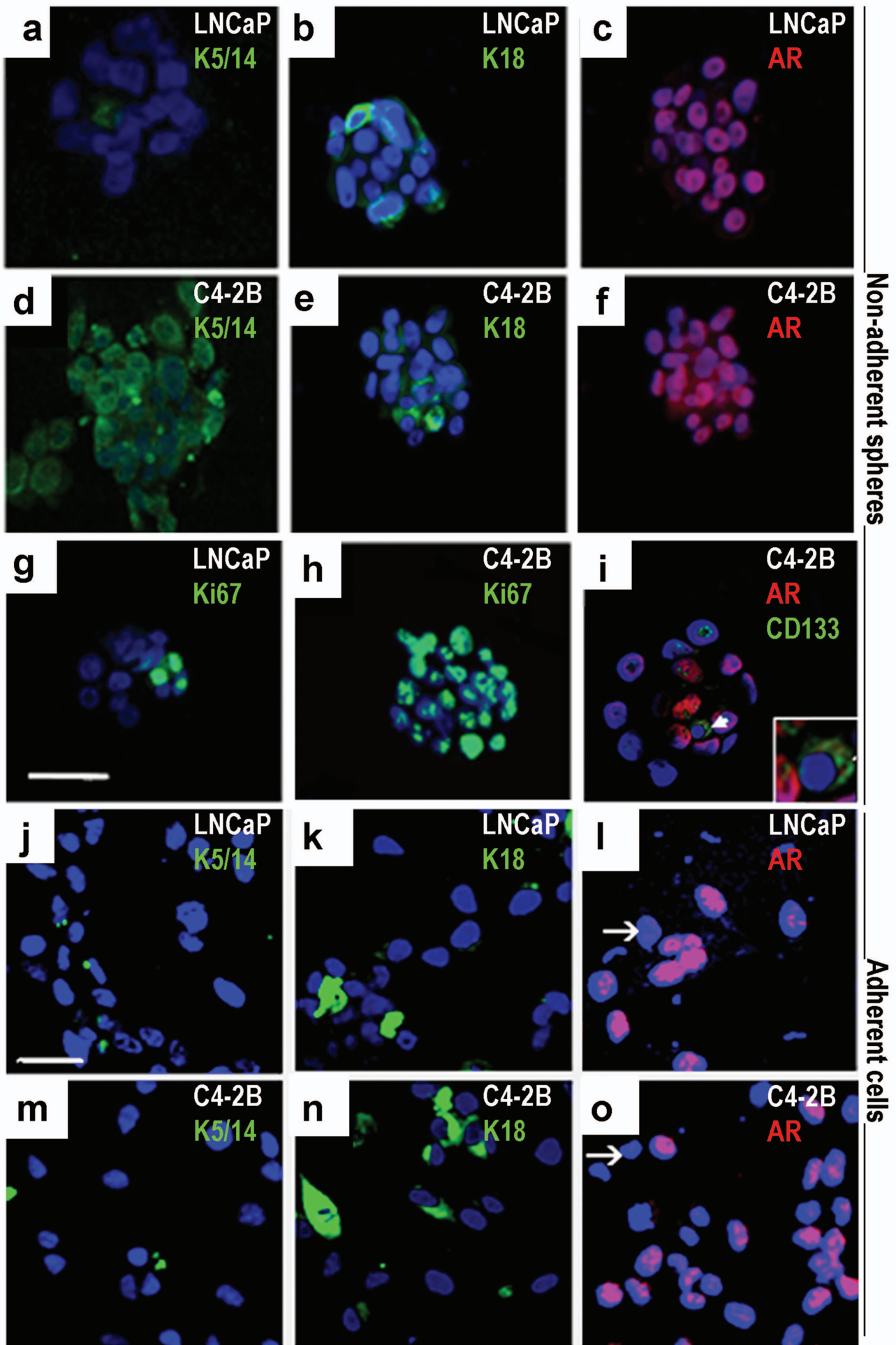
$\beta$ -catenin (t-test:  $*p < 0.0001$ ) and number of cells expressing moderate-high levels nuclear  $\beta$ -catenin (Chi-squared:  $^{\wedge}p < 0.005$ ) were observed following Wnt3a treatment.

Figure 7. Wnt3a treatment induces CD133 and inhibits AR expression in the presence of baicalutamide. Expression of CD133 in C4-2B spheres: (a) control, (b) Wnt3a, (c) Bicalutamide(Bic) and (d) Bic-Wnt3a for 7 days; CD133 (green) and DAPI nuclear counterstaining (blue), scale bar 50  $\mu$ m. (e) Number of positive cells counted per sphere in C4-2B control, C4-2B+Wnt3a, C4-2B+ Bic and C4-2B+Bic+Wnt3a. The results are the mean and standard deviation of 6-8 spheres per group ( $*p < 0.05$ ). Expression of AR in C4-2B spheres: (f) control, (g) Wnt3a, (i) Bic and (j) Bic-Wnt3a for 7 days; AR (red) and DAPI nuclear counterstaining (blue), scale bar 50  $\mu$ m. (j) Percentage of AR positive cells counted per sphere in C4-2B control, C4-2B+Wnt3a, C4-2B+ Bic and C4-2B+Bic+Wnt3a. The results are the mean and standard deviation of 4-8 spheres per group, ( $* p = 0.05-0.005$ ;  $** p < 0.005$ ).

Figure 8. A schematic model of self-renewing cancer cells and their progeny. WNT signalling regulates the self-renewal of cells with stem cell characteristics and the proliferation/differentiation of transit amplifying cells. While AR activity drives the proliferation/differentiation of transit amplifying cells. Interaction between the pathways is also possible as  $\beta$ -catenin (an effector of WNT signalling) can interact with the AR.

**Figure 1**

**Figure 2**



Non-adherent spheres

Adherent cells

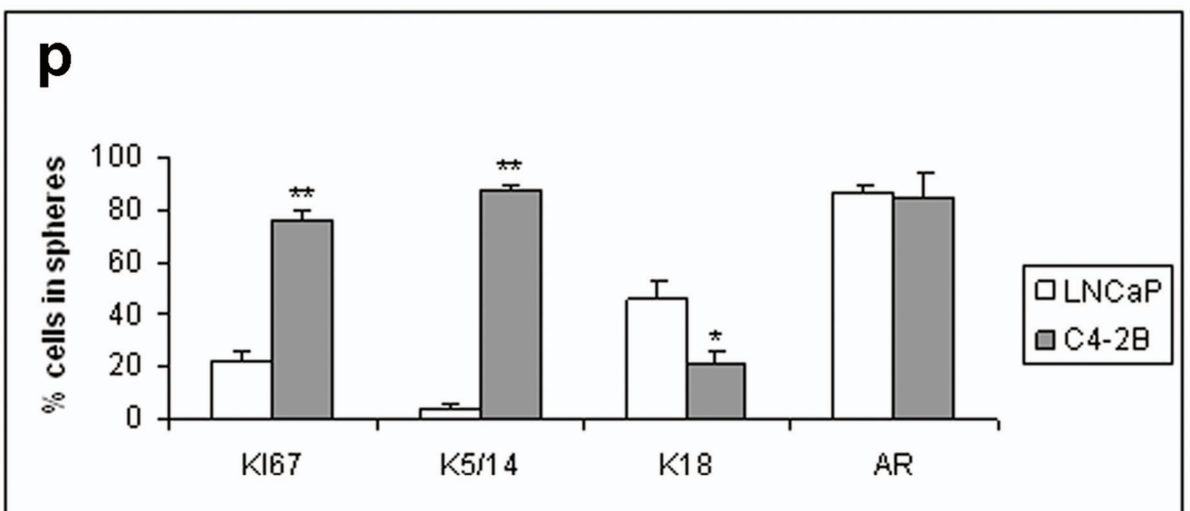
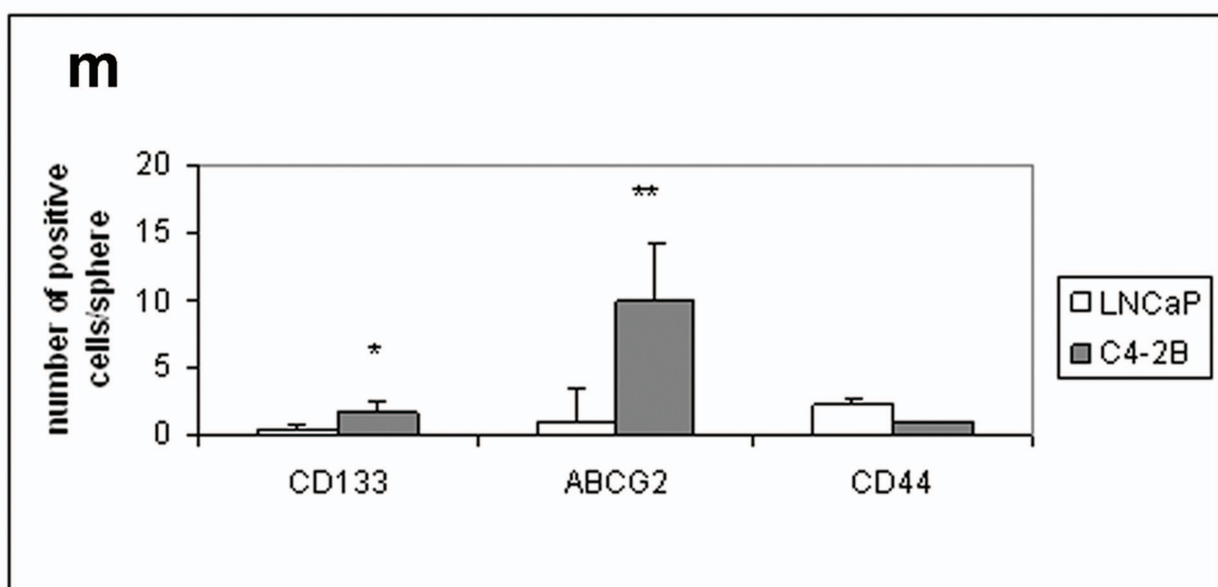
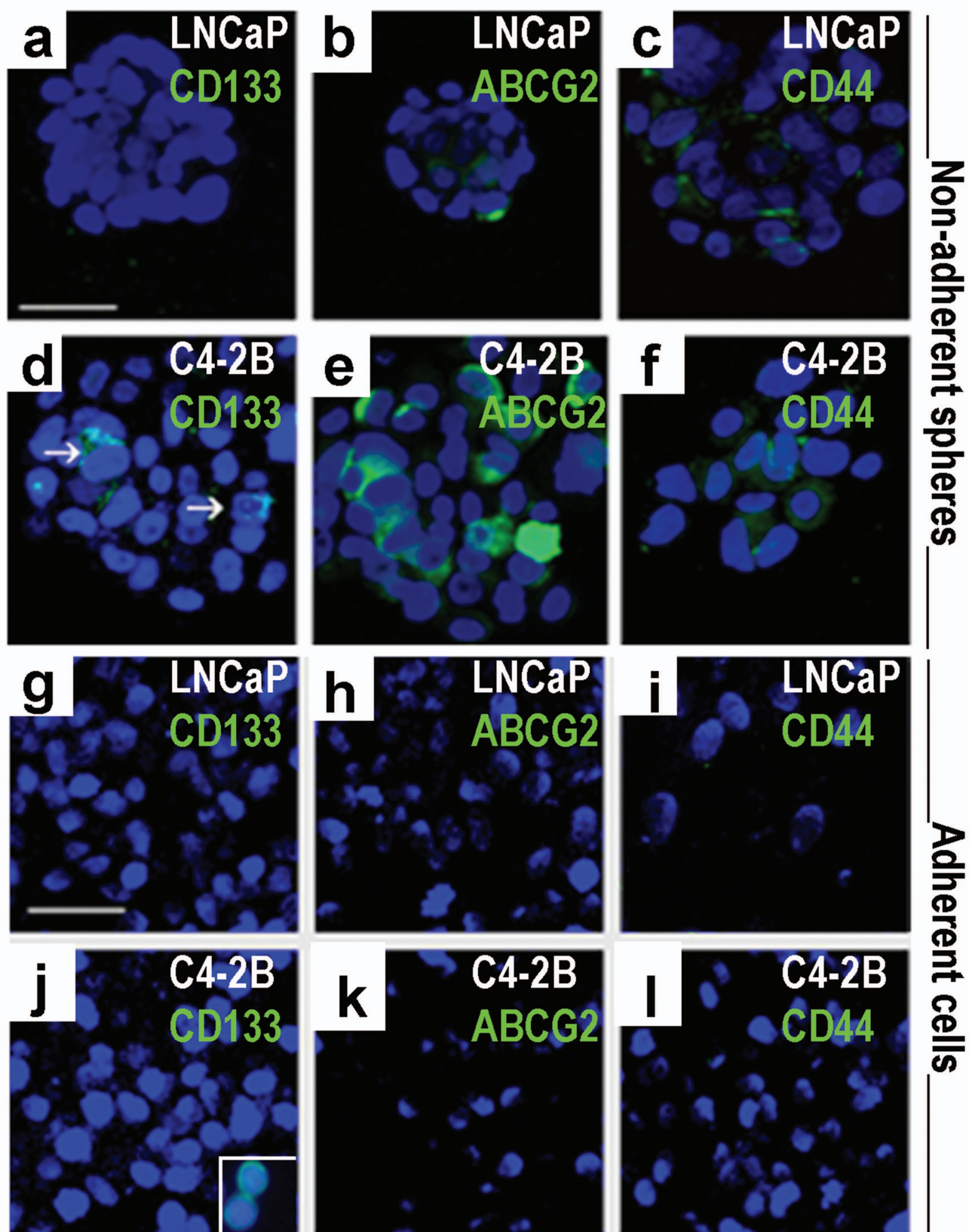
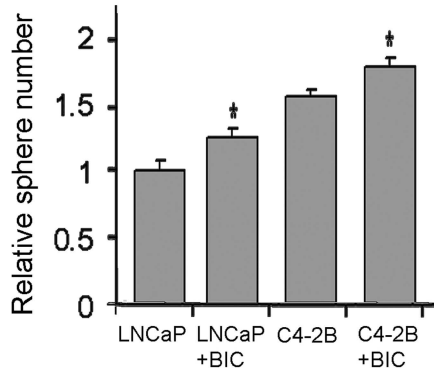
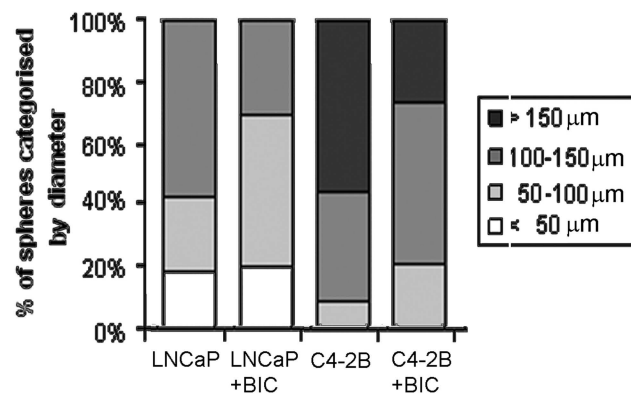
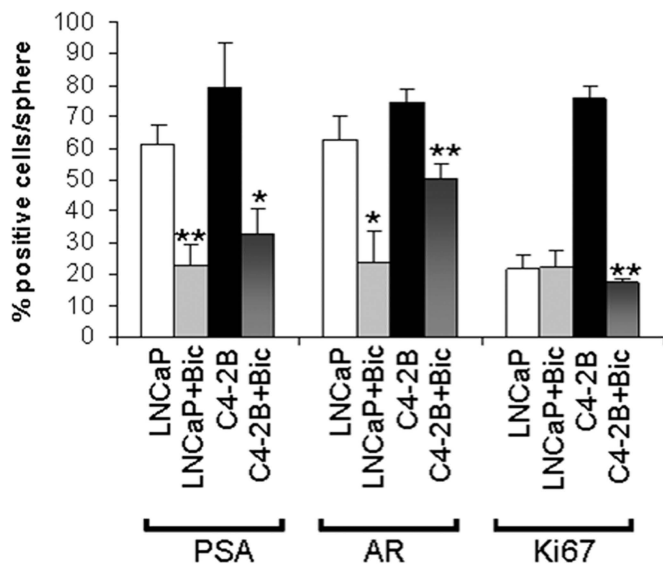
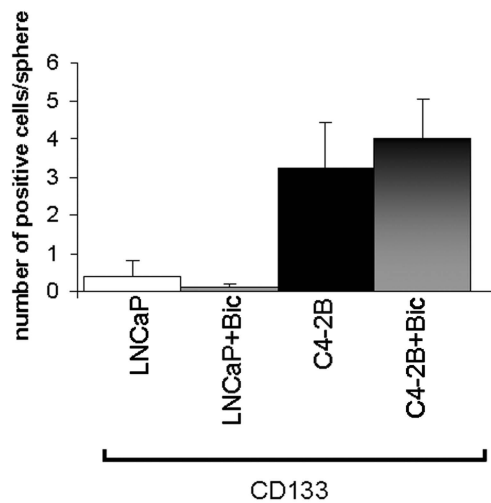


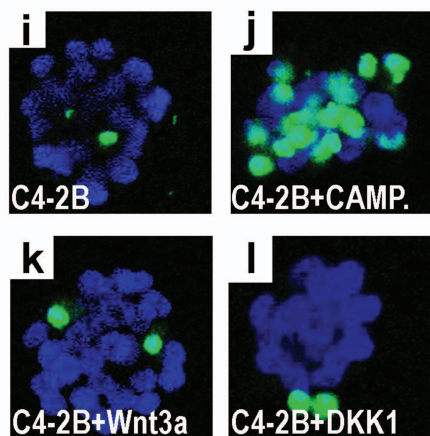
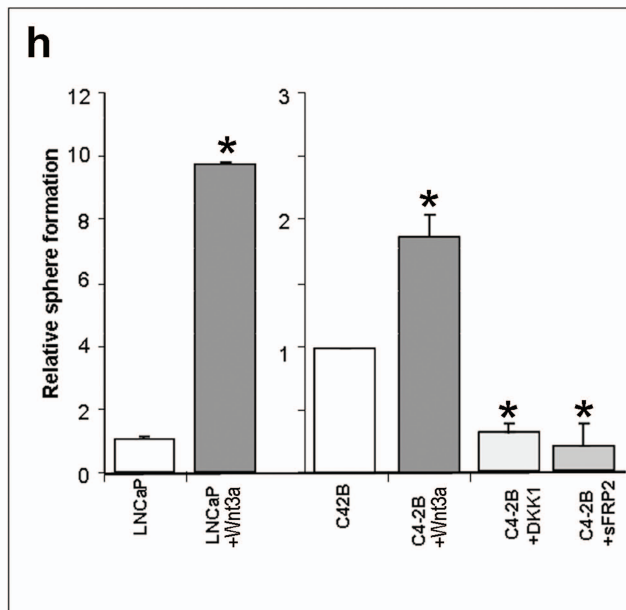
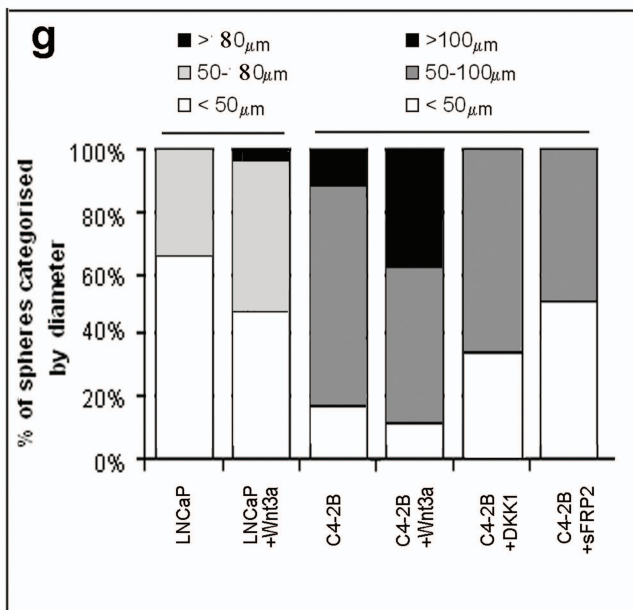
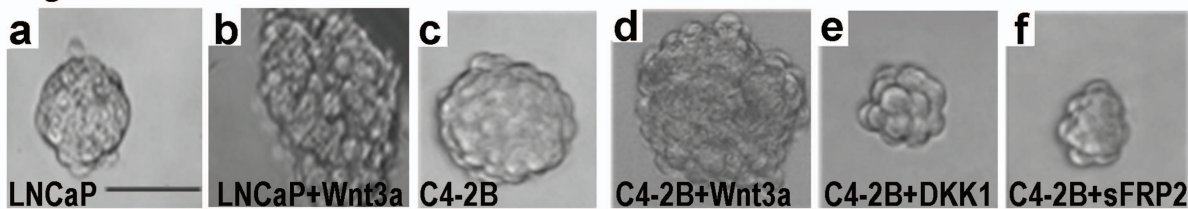
Figure 3



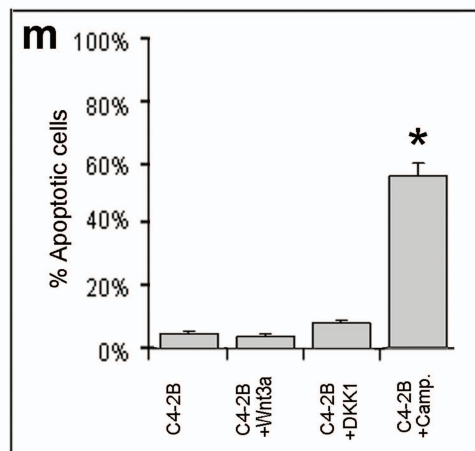
**Figure 4****a****b****c****d**



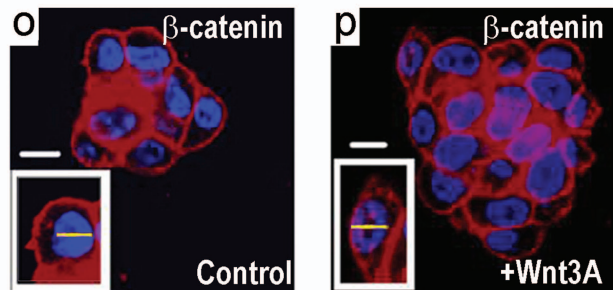
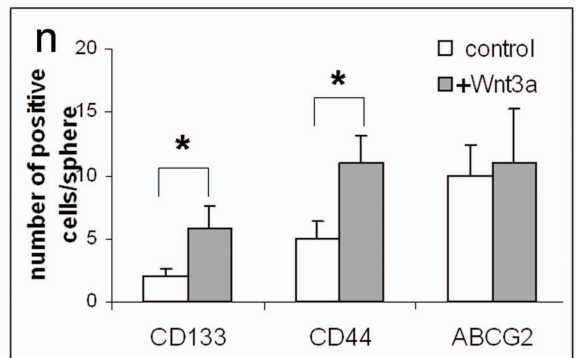
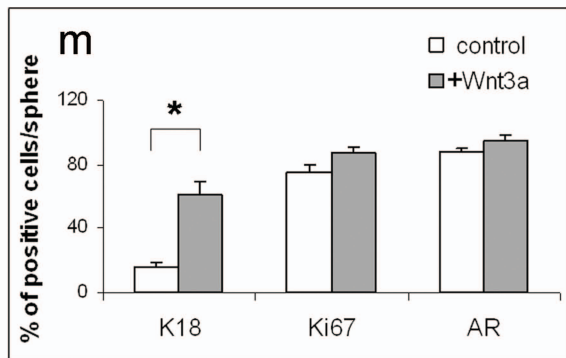
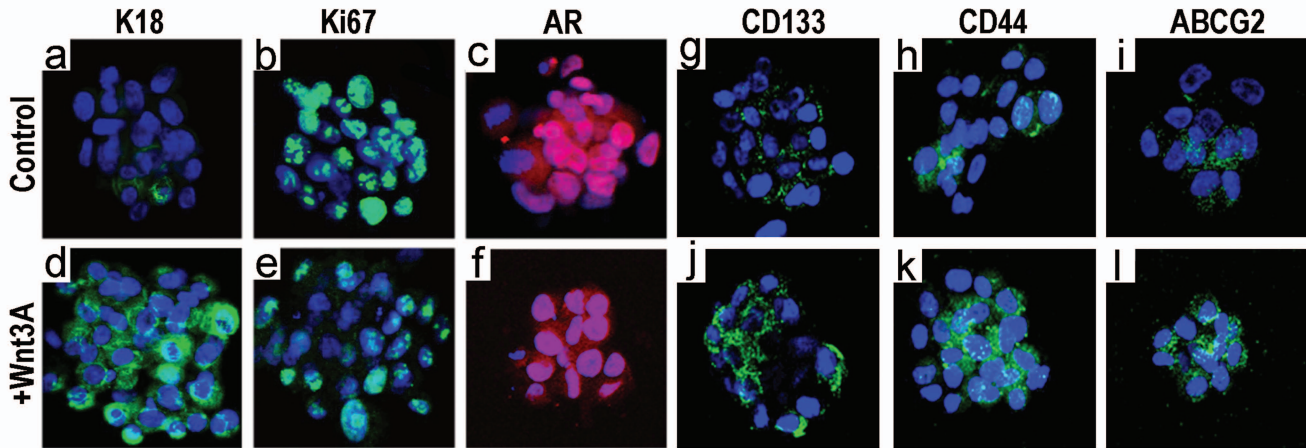
**Figure 5**



Tunel: C4-2B spheres



**Figure 6**



**q** Table showing the number of spheres, number of cells, mean nuclear fluorescence value, and nuclear fluorescence range for control and +Wnt3A conditions.

Condition	Number spheres	Number cells	Mean nuclear fluorescence value*	Nuclear fluorescence range^		
				0-74	75-149	150+
C4-2B control	7	28	63.6	19	8	1
C4-2B +Wnt 3A	6	29	120.1	8	13	8

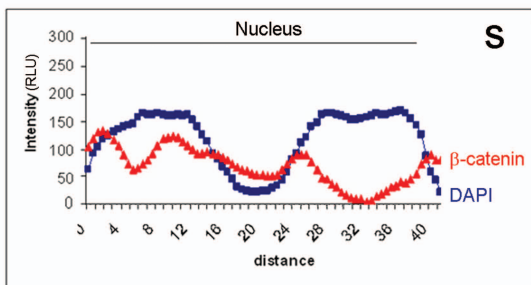
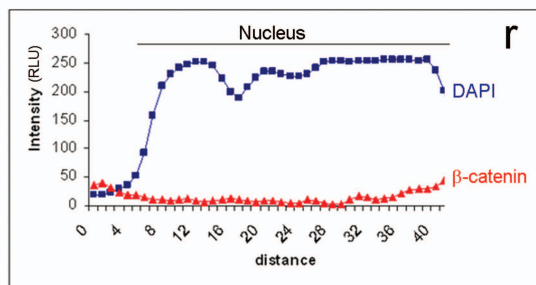
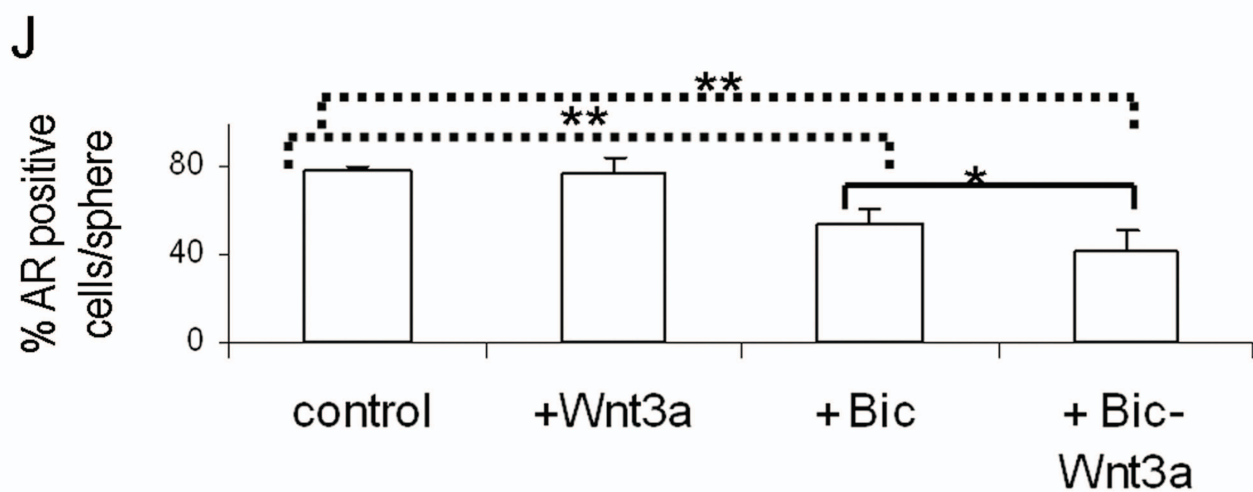
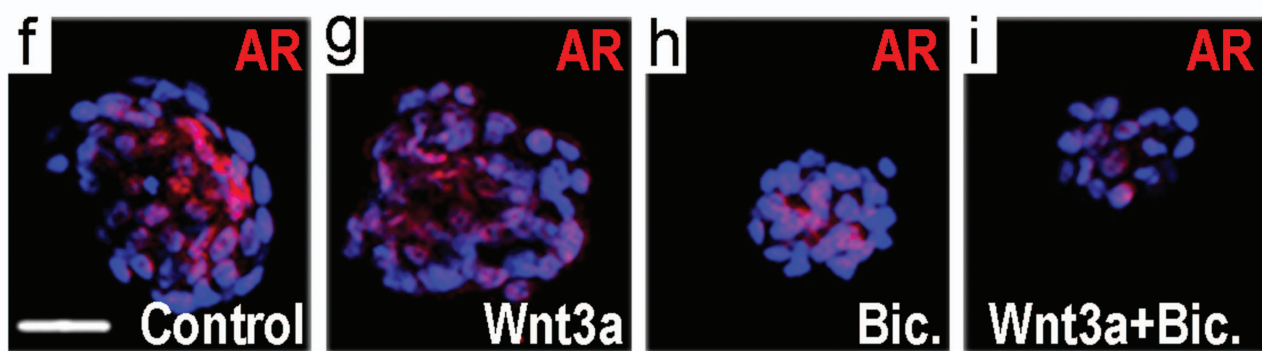
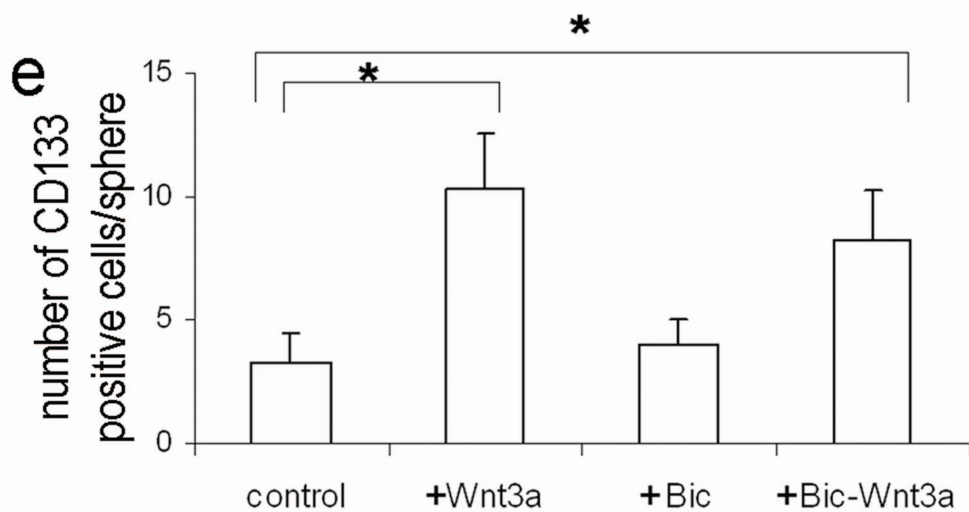
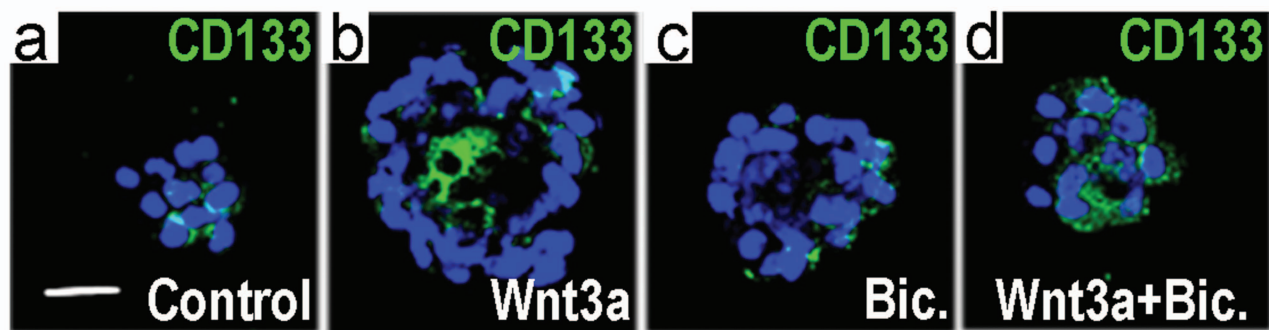




Figure 7



**Figure 8**

Self-renewing  
cancer cell

Transit amplifying  
cancer cells

Differentiated  
cancer cells

