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Miftakhova, Regina, Hedblom, Andreas, Semenas, Julius et al. (9 more authors) (2016) Cyclin A1 and P450 aromatase promote metastatic homing and growth of stem-like prostate cancer cells in the bone marrow. *Cancer research*. pp. 2453-2464. ISSN: 1538-7445

<https://doi.org/10.1158/0008-5472.CAN-15-2340>

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Cyclin A1 and P450 aromatase promote metastatic homing and growth of stem-like prostate cancer cells in the bone marrow

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The authors disclose no potential conflicts of interest

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Running title: stem-like prostate cancer cells in bone marrow metastases

Grant Support: This work was supported by the Swedish Cancer Foundation, the National Research council, the Government Health Grant, the Malmö Cancer Foundation, the Skåne University Hospital Foundation to JLP.

Word count: 5440

Abstract

Bone metastasis is a leading cause of morbidity and mortality in prostate cancer (PCa). While cancer stem-like cells have been implicated as a cell of origin for PCa metastases, the pathways which enable metastatic development at distal sites remain largely unknown. In this study, we illuminate pathways relevant to bone metastasis in this disease. We observed that cyclin A1 (CCNA1) protein expression was relatively higher in PCa metastatic lesions in lymph node, lung, and bone/bone marrow. In both primary and metastatic tissues, cyclin A1 expression was also correlated with aromatase (CYP19A1), a key enzyme that directly regulates the local balance of androgens to estrogens. Cyclin A1 overexpression in the stem-like ALDH^{high} subpopulation of PC3M cells, one model of PCa, enabled bone marrow integration and metastatic growth. Further, cells obtained from bone marrow metastatic lesions displayed self-renewal capability in colony forming assays. In the bone marrow, Cyclin A1 and aromatase enhanced local bone marrow-releasing factors, including androgen receptor, estrogen and matrix metalloproteinase MMP9 and promoted the metastatic growth of PCa cells. Moreover, ALDH^{high} tumor cells expressing elevated levels of aromatase stimulated tumor/host estrogen production and acquired a growth advantage in the presence of host bone marrow cells. Overall, these findings suggest that local production of steroids and MMPs in the bone marrow may provide a suitable microenvironment for ALDH^{high} PCa cells to establish metastatic growths, offering new approaches to therapeutically target bone metastases.

Introduction

Malignant stem cells may arise within the normal stem cell population or through carcinogenic transformation of a differentiated cell type enabling the adoption of stem like features (1, 2, 3). It is believed that only a subset of cells, termed cancer stem cells, are capable of initiating metastatic dissemination to distant organs (3-6). Cancer stem cells have been identified in malignancies including leukemia, brain, breast, colon and prostate cancers (PCa) as well (2,4,7-12).

In PCa, only rare, phenotypically distinct PCa tumor-initiating cells, also termed stem-like PCa cells have the capacity to form new tumors (4,7-9). Metastatic PCa remains a major clinical challenge, as most of PCa patients die from bone metastases (13,14). PCa are believed to favor the bone marrow, which are the unique sites enriched with hematopoietic cells, osteoblasts, stromal, extracellular matrixes and multiple cytokines (5,13,15,16). The stem-like PCa cells are believed to produce high levels of chemokine receptors which enable interaction of PCa with the infiltrated bone marrow cells to facilitate distant metastases (17,18, 19). The growth and metastases of stem-like PCa cells are also influenced by various types of steroid hormones including androgens and estrogens and their receptors (20-24). Recent reported studies suggest that estrogen and its receptor ER α are present in the hematopoietic stem cells (HSC) and bone cells (25-27). However, it remains largely unknown whether the steroid hormones may promote metastatic growth of the stem-like PCa cells in the bone marrow.

Aromatase is a steroid biosynthesis enzyme which is encoded by CYP19A1 gene, and catalyzes the conversion from androgens to estrogen (28). Aromatase expression in prostate epithelial cells and the infiltrated inflammatory cells is induced by the cytokines, and is associated with the progression of prostate cancer (29). CYP19A1 expression is elevated in prostate cancer metastatic tissue with 30-fold higher than that in primary tumors (20,22,30).

Conversely, deletion of *CYP19A1* alleles reduces incidence of PCa in mice after exposure to testosterone and estrogen (20).

The cell surface markers that recognize cancer stem cells provide possibility to study the unique mechanisms underlying cancer metastases in mouse models (31,32). Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes and has emerged as an ideal marker for isolation of stem-like cells from heterogeneous tumors (33-36). As the ALDH activity is increased in cancer stem cell populations isolated from multiple myeloma, acute myeloid leukemia (15), breast cancer lung, head and neck, gastric and colorectal cancers and recently in PCa (37-46), it is therefore considered as a reliable marker for isolation of cancer stem cells. Importantly, isolated ALDH^{high} stem-like PCa cells initiate growth of tumors in orthotopic mouse models (41).

Cyclin A1 is an important cell cycle regulator and its expression is elevated in PCa (47,48). It acts as a co-regulator of AR on VEGF promoter activity to regulate VEGF expression (47,48). Induced overexpression of cyclin A1 in PC3 cells initiated metastatic growth in lymph node, lung and liver in subcutaneous and orthotopic xenograft mouse models (48). In metastatic PC3 cells, cyclin A1 is functionally associated with VEGF and MMP2/MMP9 (48). Recently, we report that cyclin A1 interacts with ER α to promote breast cancer progression in xenograft mouse model (49). Cyclin A1 function is required for hematopoietic stem and progenitor cells (HSPC) to home to their bone marrow niches (50). In this study, we showed that ALDH^{high} stem-like cells facilitate metastatic growth by utilizing cyclin A1 and aromatase to increase androgen to estrogen conversion and recruiting extracellular matrix metalloproteinases (MMP9) from the host bone marrow.

Materials and Methods

Tissue Specimens, Tissue Microarrays, cDNA Microarrays, and CGH Arrays.

Tissue microarrays containing primary PCa (n=17) and metastatic PCa lesions (n=43) from 14 PCa patients, and paired BPH (n=48) vs. PCa tissues (n=48) from 48 patients were constructed at Department of Clinical Pathology and Cytology, Skåne University Hospital, Malmö. The mRNA expression data of cyclin A1 and aromatase (CYP19A1) were extracted from the cBioPortal database with GEO accession number: GSE21032 as described (51,52). The study was approved by the Ethics Committee, Lund University, and the Helsinki Declaration of Human Rights was strictly observed.

Immunohistochemistry Analysis

Immunohistochemistry on tumor tissue arrays was performed as previously described (47). The staining procedure was performed using a semiautomatic staining machine (Ventana ES, Ventana Inc., Tucson, AZ) as previously described (48).

Cell culture and Generation of vectors and stable and transient transfection

An androgen-insensitive cell line, PC3M cells were kindly provided by Dr. J Fidler (Department of Urology, MD Anderson Cancer Center, Houston, Texas, USA) (53). The cell lines were authenticated by the suppliers. The PC3M cells were received 2011, and fresh-frozen stocks were used. The total span of years in using PC3M cells in our labs is approximately three years. For transient transfection studies, pMSCV-cyclinA1-EGFP was generated by cloning the full-length (1.8 kb) human cyclin A1 cDNA into the *EcoRI* site of the pMSCV-EGFP construct (Clontech Inc.).

Prostate tumor spheroid formation assays

PC3M cells were cultured in polyhema-coated flasks at 5000/ml in spheroid medium modified from protocols used for mammo-sphere formation as described (54). To generate second passage spheres, cells derived from the first passage spheres were plated again at a density of 5×10^3 cells/ml and grown in culture medium for 7 days as mentioned above.

ALDEFLUOR assay

The ALDEFLUOR kit (StemCell Technologies, Vancouver, Canada) was used to isolate the population with a high ALDH enzymatic activity (stem-like ALDH^{high} cells) according to manufacturers' protocol. Cells were stained with 7AAD (BD Biosciences, San Jose, CA, USA) to distinguish viable cells from dead cells and analysis on CyAn™ ADP flow cytometer or FACS Aria (Beckman Coulter). FACS data were analyzed using FCS Express software (DeNovo Software, CA).

Mouse model of PCa distant metastases

The animal studies were approved by the Swedish Regional Ethical Animal Welfare Committee and the guidelines were strictly followed. Athymic NMRI nude male mice aged 8–12 weeks (Taconic Europe, Denmark) were used. For intravenous injection of tumor cells, mice were sub-lethally irradiated with two doses of 2 Gy administered 2 hours apart using a ¹³⁷Cs source at a dose rate of 1Gy/min as described (55). Equal amount of ALDH^{high} cells (1×10^5 cells /mouse) or unsorted PC3M cells (2×10^6 cells/mouse) were injected into mice via tail-veins. For intracardiac injection, 1×10^5 cells /mouse were injected into the left ventricle of anaesthetized mice (1% isoflurane through inhalation). *In vivo* imaging device (IVIS imaging system, PerkinElmer). For HLA-ABC antibody-based imaging, mice were injected intraperitoneally with 30 µg of HLA-ABC antibody conjugated with 680 DyLight NHS-ester (LifeTechnologies) (56).

Identification of metastatic PCa cells in the bone marrow of mice by FACS

To identify metastatic PCa cells in the bone marrow of irradiated recipients after transplantation, bone marrow cells from long bones of the recipient mice were isolated and were assessed using FACS methods as described (50).

Evaluation of ability of single cells from metastatic tumors of mice to form colony-forming unit using methylcellulose-based medium

To assess the repopulating ability and differentiation potential of the BM progenitor cells or metastatic PCa cells in the bone marrow, we used a methylcellulose-based colony-forming assay according to the manufacturer's description (MethoCult™, Stem Cell Technologies). Colonies containing 30 and more cells were scored after 14 days.

Immunofluorescence analysis

For immunofluorescence analysis, cell suspensions were fixed on slides in 4% paraformaldehyde (Merck KGaA, Germany) for 15 minutes at room temperature (RT). The slides were stained with primary antibodies including anti-HLA (Biosite) and anti-cyclin A1 (BD Pharmingen).

Treatment of ALDH^{high} or ALDH^{low} cells with 17- β -estradiol or DTH

For estrogen treatment, ALDH^{high} or ALDH^{low} populations were sorted from PC3M cells and were maintained in 10% charcoal stripped phenol red free medium (CSS) (Gibco, Life Technologies) for 24 hour prior to treatment with 17- β -estradiol at 10 nM and DTH at 5 nM (Sigma-Aldrich Inc.). The effects of 17- β -estradiol and DTH on ALDH^{high} or ALDH^{low} cells

were determined using the nonradioactive tetrazolium dye-based proliferation assay (MTS) (Promega Biotech) according to the manufacturer's protocol.

Co-culture of ALDH^{high} or ALDH^{low} cells with the bone marrow and measurement of estradiol production

For MTS assay, ALDH^{high} or ALDH^{low} cells were cultured in serum-free phenol red free RPMI medium containing 50% of the bone marrow extracts of NMRI-nude mice. The cells were cultured for 48 hours and were subjected for MTS assay. For measurement of estradiol production, Estradiol Assay kit (R&D Systems Ltd, Abingdon, UK) was used according to the manufacturer's protocol. For treatment with Aromatase inhibitor, Type I aromatase inhibitor (Merck Millipore) was used at 100 nM concentration. Concentrations were determined by measuring absorbance on an Infinite M200 multimode microplate reader (Tecan Sunrise). Testosterone and estradiol levels were determined using an immunoassay from Roche Diagnostics (Estradiol III) on a Cobas 6000 Analyzer (Roche Diagnostics, USA).

Statistical analysis

Statistical analysis was performed. Distribution of overall survival (OS) was estimated by the method of Kaplan-Meier, with 95% confidence intervals. Differences between survival curves were calculated using the log-rank test using statistical program (SPSS, 16.0).

Description of experimental procedures and methods are given in Supplemental Materials and Methods (Supplemental Information).

Results

Expression of cyclin A1 and aromatase in patients with PCa

Immunohistochemical analysis was performed to assess protein expression of cyclin A1 and aromatase on tissue microarrays (TMAs) consisting of benign prostatic hyperplasia (BPH) (n=48 patients), primary PCa (n=65) and metastatic PCa in lymph nodes, bone marrows and lungs (n=43) from 14 PCa patients who suffered bone metastasis. Cyclin A1 expression was predominantly nuclear, while aromatase expression was cytoplasmic in primary and metastatic PCa tissues (Figure 1A). Expression of cyclin A1 was higher in primary PCa than in BPH (for cyclin A1, $p < 0.001$) (Figure 1B). Metastatic lesions displayed increased cyclin A1 expression compared with that of primary PCa ($p = 0.019$) (Figure 1C). Although aromatase expression did not show significant increase in metastatic PCa compared with primary tumors (Figure 1C), it was significantly higher in primary PCa than in BPH ($p = 0.001$) (Figure 1B). Further, expression of cyclin A1 was positively correlated with aromatase expression in both primary and metastatic PCa as determined by Spearman's rank correlation test ($R^2 = 0.398$, $p < 0.001$).

We next examined mRNA expression of cyclin A1 and aromatase using a PCa cohort consisting of primary (n=131) and metastatic (n=19) PCa tissues extracted from the public available databases (51). Cyclin A1 and aromatase mRNA expression remained to be similar between primary and metastatic PCa tissues (Figure 1D). Somatic mutations or amplifications in genes encoding cyclin A1 and aromatase appeared to be rare. Only 4% (8 out of 216) amplifications in gene encoding for cyclin A1 and 2% (4 out of 216) amplifications in *CYP19A1* gene were observed (Figure 1E). This suggests that overexpression of cyclin A1 and aromatase in PCa may be due to the alterations at protein levels. Nevertheless, we observed that a subset of patients with higher *CYP19A1* expression suffered poorer BCR-free survival as compared with those with lower *CYP19A1* ($p < 0.001$) (Figure 1F). Cyclin A1

expression was not associated with disease-free survival of PCa patients in these two cohorts ($p=0.348$) (Figure 1F).

Cyclin A1 promoted growth of “tumor spheres” in vitro

The stem-like ALDH^{high} and non-stem-like ALDH^{low} subpopulations of PC3M cells were sorted by FACS using the ALDEFLUOR assay (Figure 2A and B), and were examined for the expression of cyclin A1 and aromatase. Both ALDH^{high} and ALDH^{low} cells expressed cyclin A1 and aromatase (Figure 2C). Next, the role of cyclin A1 in tumor-initiating property of stem-like cells was assessed. We employed 3-dimension (3-D) culture systems, as this method is well-established for assessment of breast cancer stem cells (57, 58). PC3M cells that were stably transfected with pMSCV-EGFP or pMSCV-EGFP-A1 were seeded in 3-D medium and were cultured for a period of 7 days. One of the characteristics of the stem-like cells is the ability to form “tumor spheres”. PC3M cells expressing cyclin A1 formed larger size of “tumor spheres” with increased cell numbers as compared with the controls ($p=0.03$) (Figure 2D and E). We next re-plated the single cell suspensions prepared from the spheres to test their self-renewal ability. The re-plated cells expressing cyclin A1 again formed larger spheres with higher cell numbers compared with the controls ($p=0.015$) (Figure 2D and E). Further, PC3M cells expressing cyclin A1 contained higher frequency of ALDH^{high} subpopulation ($5.43\pm2.69\%$) with approximately 3-fold increase compared with the controls ($1.89\pm0.85\%$) ($p<0.001$) (Figure 2F). This data suggests that PC3M cells contain stem-like cells and that cyclin A1 overexpression increases frequency of the tumor-initiating cells *in vitro*.

Cyclin A1 and aromatase promote metastatic dissemination of ALDH^{high} stem-like cells to the bone marrow in mouse xenografts

To investigate whether the ALDH^{high} stem-like cells may invade to the bone marrow, we established an *in vivo* mouse model. Nude mice were sub-lethally irradiated with low dose γ -irradiation to inhibit host immune function prior to the transplantation of the tumor cells. We observed that expression of aromatase and ER α was higher in ALDH^{high} cells overexpressing cyclin A1 compared with the controls (Figure 3A). Equal amount of ALDH^{high} subpopulations sorted from PC3M cells expressing the vectors were introduced into the irradiated mice via intravenously injection (Figure 3B). The human HLA-ABC antibody conjugated with fluorescent dye was administrated into the mice for bioluminescent *in vivo* imaging analysis (Figure 3C). Tumors that were positive to HLA-ABC fluorescent antibody were detected in both groups of mice using imaging device after 80 days post-transplantation (Figure 3C). We performed FACS analysis and confirmed that the transplanted cancer cells positive to HLA-ABC integrated into the bone marrow (Figure 3D). There was clear trend that a higher frequency of ALDH^{high} cells overexpressing cyclin A1 homed the host bone marrow compared with the controls (n=4 mice/per group) (P=0.076) (Figure 3D).

Since the FACS analysis is dependent on the quality of HLA-ABC antibody and its ability to bind tumor-producing HLA-ABC antigens *in vivo*, therefore this method represents certain difficulties with large variations. We therefore examined expression of cytokeratins that specifically recognize human PCa cells in the bone marrow (Figure 3E). A significantly higher number of cytokeratins-positive cells and Ki-67-positive cells was observed in the bone marrow of mice which received ALDH^{high} cells expressing cyclin A1 vector (for cytokeratins, $p=0.024$; for Ki-67, $p<0.001$) (Figure 3E, F, G and H). These data showed that ALDH^{high} cells integrated into the bone marrow and that cyclin A1 overexpression enhanced metastatic growth of the ALDH^{high} cells.

We transplanted unsorted PC3M cells expressing cyclin A1 or EGFP vector into the irradiated mice via intravenous injection. Metastatic tumors in prostate, lung and liver appeared in 3 out of 5 mice which received PC3M cells expressing cyclin A1, while a large subcutaneous tumor was formed near the proximal end of the tail in 1 out of 5 control mice (Supplemental Figure 1). There was a clear trend that elevated levels of cyclin A1 in tumors were positively correlated with poorer overall survivals of the xenograft mice (Supplemental Figure 1). No metastases to the bone marrow were detected in these xenograft mice. We therefore induced unsorted PC3M cells expressing cyclin A1 or EGFP vectors into mice via intracardiac injection which allows tumor cells entering directly into systemic circulation, and homing to the bone marrow. Tumor metastases were detected in both groups of mice after 33 days post-transplantation (5 mice/group) using bioluminescent *in vivo* imaging analysis (Supplemental Figure 2A and B). FACS analysis revealed that HLA-ABC-positive PC3M cells integrated into the bone marrow (Supplemental Figure 2C and D). Overexpression of cyclin A1 increased frequency of PC3M homing to the bone marrow relative to controls, as determined by using cytokeratins-stainings of tumor cells ($P= 0.016$ for CK5 and $P= 0.0014$ for CK20) (Supplemental Figure 3).

Metastatic growth of ALDH^{high} PC3M cells in the bone marrow through cyclin A1 and aromatase associated pathways

We next wanted to assess whether metastatic lesions initiated by ALDH^{high} PC3M cells may retain stem-like properties such as self-renewal and differentiation. We isolated bone marrow cells from the mice bearing bone marrow metastases initiated by ALDH^{high} cells expressing cyclin A1 or control vector. The isolated bone marrow cells were then subjected to colony formation Unit (CFU) assay in the semi-solid MethoCult™ medium which is commonly used

for assessment of self-renewal and differentiation ability of the hematopoietic cells (Figure 4A). Bone marrow cells containing PCa metastatic lesions isolated from the mice (4 mice/group) formed colonies (Figure 4B). To access whether the CFUs might be derived from both host hematopoietic stem/progenitor cells, and metastatic ALDH^{high} cells, single cell suspensions were prepared from the colonies and were examined for the co-expression of cyclin A1 and HLA-ABC, a specific human tumor antigen. We observed that the subset of cells collected from the CFUs indeed co-expressed cyclin A1 and HLA-ABC, and the signals were enhanced in cells overexpressing cyclin A1 (Figure 4C). By using the combination of Giemsa staining and visualization of EGFP positivity in CFUs, we further identified CFUs derived from ALDH^{high} cells (Figure 4D, E, F and G). This suggests that the metastatic lesions derived from ALDH^{high} cells are able to self-renewal and repopulate into new tumor cells in CFU assays, and this characteristic is similar to the host bone marrow hematopoietic stem/progenitor cells. Further, we observed that there was a higher proportion of PCa cells in the CFUs derived from the bone marrow samples of mice bearing metastases with elevated level of cyclin A1 as compared with the controls ($p < 0.001$) (Figure 4H). These new findings showed that the metastatic lesions derived from ALDH^{high} PC3M cells retained self-renewal and repopulating properties.

We examined the expression of aromatase and ER α in the CFUs derived from the metastatic bone marrow of the xenograft mice. Expression of aromatase and ER α retained in the CFUs-derived from the metastatic lesions initiated by the ALDH^{high} cells, and was enhanced by overexpression of cyclin A1 (Figure 4I and J). The absence of both aromatase and ER α in the CFUs of tumor-free bone marrow was observed (Figure 4 I and J). In contrast, AR expression was absent in the CFUs derived from the metastatic lesions but was present in the CFUs of tumor-free bone marrow (Figure 4K), suggesting that AR is produced by the host bone

marrow microenvironment. We observed that overexpression of cyclin A1 was positively associated with the decreased apoptosis, as determined by examining the expression of cleaved caspase-3 in the CFUs derived from the metastatic PCa lesions (Figure 4L).

Cyclin A1 overexpression in ALDH^{high} cells increased MMP9 expression in the bone marrow cells

We further examined aromatase expression in series bone marrow sections of the xenograft mice bearing metastases using immunohistochemical analysis. Approximately 10% aromatase-positive cells were observed in the metastatic bone marrow of control xenograft mice (Figure 5A and B). Overexpression of cyclin A1 increased aromatase-positive cells by 60% ($p<0.001$) and staining intensity of aromatase (Figure 5A, B and C). The integration of ALDH^{high} cells overexpressing cyclin A1 into the bone marrow significantly increased MMP9 expression in the host bone marrow ($p=0.016$) (Figure 5D, G and H). Similar to AR, MMP9 expression was absent in ALDH^{high} cells, but was present in the host bone marrow cells (Figure 5E and F). These data suggest that ALDH^{high} cells with elevated level of cyclin A1 influence the bone marrow-releasing factor such as MMP9.

ALDH^{high} stem-like subpopulation of PC3M cells are more sensitive to aromatase inhibition than the ALDH^{low} cells

We further investigated whether the ability of ALDH^{high} cells to initiate metastatic growth in the bone marrow is related to their stem-like characteristics with growth advantages. We subjected equal amount of ALDH^{high} and ALDH^{low} cells sorted from PC3M cells expressing EGFP-cyclin A1 or control vectors to the MTS-based proliferation assays. The growth rate of ALDH^{high} subpopulation was significantly higher with 20% increase compared with the ALDH^{low} cells ($p=0.012$) (Figure 6A). Overexpression of cyclin A1 increased growth of

ALDH^{high} cells by 60% as compared with the ALDH^{low} cells ($p < 0.001$) (Figure 6A). Further, overexpression of cyclin A1 increased growth rate only in ALDH^{high} cells ($p = 0.012$) but not in ALDH^{low} cells (Figure 6A). This suggests that ALDH^{high} cells with cyclin A1 overexpression have growth advantages over that of ALDH^{low} cells. Next, we inhibited aromatase production in ALDH^{high} vs. ALDH^{low} cells using an irreversible type I aromatase inhibitor. Inhibition of aromatase reduced proliferation of control ALDH^{high} cells ($p = 0.008$) and ALDH^{high} cells overexpressing cyclin A1 ($p < 0.001$) (Figure 6B), but had no significant effect on ALDH^{low} cells. We further examined the effect of bone marrow microenvironment on the growth of ALDH^{high} vs. ALDH^{low} cells. We co-cultured equal amount of the sorted cells with the bone marrow extracts of mice. ALDH^{high} cells displayed higher ability to adapt and grow in the bone marrow-containing medium compared with the ALDH^{low} cells as determined by MTS proliferation assays (Figure 6C). Overexpression of cyclin A1 significantly enhanced ability of ALDH^{high} cells, but not that of ALDH^{low} cells to grow in the bone marrow-containing medium (Figure 6C). Next, we measured the concentration of estradiol, an end product of androgen to estrogen conversion, in ALDH^{high} PC3M cells, in bone marrow cells alone and in co-cultures of ALDH^{high} PC3M cells and bone marrow cells. Estradiol levels in ALDH^{high} PC3M cells cultures were similar to that in the medium only (RPMI1640 containing 10%FBS) (Figure 6D), suggesting that ALDH^{high} PC3M cells do not produce detectable levels of estrogen *in vitro*. Bone marrow cells produced detectable levels of estradiol, which were significantly increased in co-culture with ALDH^{high} (Figure 6D and E). Overexpression of cyclin A1 further increased estradiol production (for BM alone vs. co-culture of BM and ALDH^{high} cells expressing EGFP, $P < 0.001$; for co-culture of BM and ALDH^{high} cells expressing EGFP-A1 vs co-culture of BM and ALDH^{high} cells expressing EGFP, $P = 0.029$) (Figure 6D). Inhibition of aromatase by aromatase inhibitor in ALDH^{high} cells overexpressing cyclin A1 prior to the co-culture with the bone marrow cells in serum-

free medium led to a significantly reduction in estradiol level in the co-culture ($p < 0.001$) (Figure 6E).

Discussion

Increasing evidence has suggested that cancer stem cells are the cell origin to initiate distant metastases (4,5,59). However, the unique cellular pathways that enable cancer stem cells to establish metastatic lesions into the distant organs remain largely unknown. In this study, we observed that cyclin A1 protein expression was significantly higher in PCa metastatic lesions from PCa patients. In agreement with the previous reported study (60), our data also showed that aromatase was abnormally expressed in human PCa. Expression of both cyclin A1 and aromatase proteins was significantly higher in primary PCa than in BPH. A subset of patients with upregulated aromatase mRNA expression suffered poorer BCR-free survival, although this finding was limited to fewer patients. Nevertheless, our present study adds new information on the clinical importance of aromatase expression in PCa progression.

In this study, we found that ALDH^{high} subpopulation of PC3M cells had higher growth ability compared with ALDH^{low} subpopulation. It has been shown that γ -radiation at low dose (2-4 Gy) promoted tumor cell infiltration with no pronounced impact on host immune function in nude mice (55). The ALDH^{high} subpopulation accounted for $1.89 \pm 0.85\%$ of total PC3M cells initiated metastatic growth in the bone marrow of the irradiated immune-suppressive host mice, whereas unsorted PC3M cells homed to the bone marrow only when tumor cells were implanted by intracardiac injection. Intracardiac injection delivers tumor cells directly into systemic circulation, while tail vein injection necessitates that tumor cells pass through of lung and liver vasculatures before reaching the bone marrow. Thus our study establishes a robust mouse model and FACS-based analysis to study metastases of cancer cells and stem-

like cancer cells to bone. Our findings are in agreement with the reported studies in breast cancer (37-39) and PCa (40,41), which suggest that rare subsets of cancer cells are sufficient to initiate metastases to distant organs.

Although ALDH^{high} PC3M cells alone are sufficient to initiate metastases into the bone/bone marrow, overexpression of cyclin A1 increased frequency of the integrated ALDH^{high} cells by 40-50%. These findings are coincident with that overexpression of cyclin A1 promoted proliferation of ALDH^{high} subpopulation, but not ALDH^{low} subpopulation. Aromatase facilitates the metabolism of testicular testosterone and androstenedione to estrogen, and directly regulates the local balance of androgens to estrogens (61). Growing evidence suggests that the microenvironment has profound effects on cancer metastases to preferential tissues/organs. Aromatase is able to utilize exogenous testosterone and extracellular factors from the tumor-associated microenvironment to produce estradiols and other hormones (29).

We found that the expression of bone marrow-releasing factors including AR, ER α , MMP9 was induced as a result of metastatic integration of ALDH^{high} cells. Our new data show that increased aromatase activity in ALDH^{high} population in the context of the bone marrow microenvironment enhances estradiol production. Moreover, incorporation of a pharmacological inhibitor establishes the requirement for aromatase in estradiol production in our model. Overexpression of cyclin A1 further increases estradiol production, providing insight into how cyclin A1 overexpression in ALDH^{high} cells can influence the tumor microenvironment in the bone marrow niches. We were unable to detect significant changes in the steady state levels of androgens in mono- or co-cultures of ALDH^{high} cells and bone marrow cells (data not included).

Our study suggests that cyclin A1 is a key factor that regulates aromatase-associated pathways. However, the precise cellular mechanisms by which cyclin A1 regulates aromatase expression remain to be further explored in future studies. Taken together, our data suggest that the stem-like PCa cells express high levels of several key factors that facilitate metastatic homing to the bone marrow environment. Our study will provide new insights into the PCa bone metastases and help to design novel targeted therapy.

Acknowledgements

The University of Nottingham and the Royal Society of London (CA), the Yorkshire Cancer Research (NJM), CRUK (DH) and Kazan Federal University (AR) are acknowledged. We thank Elise Nilsson, Nishtman Dizayi, Pradeep Kumar and Richard Karlsson for the technical support.

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Figure legends

Figure 1. Evaluation of the clinical importance of cyclin A1 and aromatase CYP19A1 in PCa patients.

(A,B,C). Immunohistochemical analysis of cyclin A1 and aromatase in primary PCa cancer specimens, and primary PCa cancer vs. PCa metastatic lesions in lymph nodes (LN), bone marrow (BM) and lung (Lung). Representative microphotographs are shown. Box-plot quantitative comparison of cyclin A1 and aromatase expression between BPH and primary cancer specimens in (B), and between primary and metastatic cancer specimens in (C). The paired Wilcoxon's rank sum test analyses are shown. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (D). Box plots presents expression of mRNA of *cyclin A1* and *aromatase* in primary and metastatic PCa. (E). Gene alteration profiles for *CYP19A1* and cyclin A1 in primary and metastatic patients. (F). Kaplan-Meier survival analysis to show biochemical recurrence (BCR). *P* values are indicated.

Figure 2. The role of cyclin A1 in self-renewal and proliferation of PCa cancer stem-like cells. (A and B). Representative FACS plots of ALDH^{high} vs ALDH^{low} cells within a total PC3M cells. (C). Immunoblot analysis of ALDH^{low} vs. ALDH^{high} cells using antibodies as indicated. (D). Quantification of numbers of cells from the “tumor spheres” of the two groups as indicated. (E). Representative microphotographs show the morphologies of the “tumor spheres”. (F). Percentages of ALDH^{high} cells expressing EGFP or EGFP-A1 are shown. SD± values indicate means of three independent experiments. * $p = 0.05$ is indicated.

Figure 3. Cyclin A1 overexpression promotes tumor metastasis by increasing proliferation and metastatic potential of ALDH-high cells.

(A). Immunoblot analysis of ALDH^{high} cells expressing pMSCV-EGFP or pMSCV-EGFP-A1 using antibodies against cyclin A1 and aromatase and ERα. (B). A schematic chart depicts the procedure of transplantation of sorted ALDH^{high} PC3M cells into irradiated mice through tail-vein injection. (C). Representative images of the bioluminescent *in vivo* imaging using IVIS

imaging device. (D). Representative FACS plots show that the percentage of HLA-ABC-positive metastatic PCa cells in the bone marrow of the two groups of mice. PC3M tumor cells were used as control as shown in the left (4 mice/group). (E and G). Representative of microphotographs of the bone marrow sections immune-stained with antibodies against cytokeratins in (E) and Ki-67 in (G). The enlarged areas are shown in the right. (F and H). The expression of cytokeratins and percentage of Ki-67 positive cells are quantified (3 mice/group).

Figure 4. Evaluate bone marrow metastasis initiated by ALDH-high PC3M cells.

(A). A schematic chart depicts the experimental procedure. (B). Colony-Forming Unit counts (3-4 mice/group). (C). Cell suspensions were collected from the colonies as mentioned in (B) and were stained with antibodies against cyclin A1 and HLA-ABC. (D, E, F and G). Giemsa staining and fluorescence analysis of EGFP-expressing CFU cells with respective higher magnification images in (F,G). (H). Percentages of EGFP-positive PCa cells in the total CFUs from each group are shown. (I, J, K and L). Immunoblot analysis of aromatase, ER α , AR and cleaved caspase-3 in CFUs derived from the bone marrows of mice received ALDH^{high} cells expressing EGFP or EGFP-A1, and from the bone marrow of irradiated tumor-free wild-type mice or cyclin A1-null mice which served as controls.

Figure 5. Cyclin A1 overexpression increases aromatase and MMP9 expression in mouse bone marrow.

Immunostaining and statistical analysis of aromatase (A,B, C) and MMP9 (D) expression in bone marrow sections from xenograft recipients of ALDH^{high} PC3M cells expressing EGFP or EGFP-A1 with tumors cells (▲) (n=3 mice/group). (E, F, G, H). Immunoblot analysis of MMP9 expression in (E) wild type mouse bone marrow, ALDH^{high} EGFP and EGFP-A1

subpopulations of PC3M cells and (F) bone marrow and bone marrow-derived CFU-derived cell suspensions from ALDH^{high} PC3M cells expressing EGPF or EGFP-A1 recipient xenograft mice. Immunoblot (G) and statistical analysis (H) of MMP9 expression in bone marrow ALDH^{high} PC3M cells expressing EGPF or EGFP-A1 recipient mice (n=3/group). * $p < 0.05$ and *** $p < 0.001$ are indicated.

Figure 6. The effect of inhibition of aromatase on ALDH^{high} and ALDH^{low} cells and their bone marrow microenvironments.

(A). The proliferation rates of equal amount of ALDH^{high} vs. ALDH^{low} PC3M cells that express EGFP or EGFP-A1. (B). Proliferation rates were determined in ALDH^{high} vs. ALDH^{low} cells that were treated with aromatase inhibitor or solvent (Ctrl). (C). Equal amount of ALDH^{high} vs. ALDH^{low} cells were co-cultured with the bone marrow extracts and proliferation of the cells were determined using MTS assays. (D). Estradiol concentrations (pg/ml) were determined in different samples as indicated. The culture medium phenol red free RPMI1640 contains 10% serum. (E). The effect of aromatase inhibitor on estradiol concentrations in different samples. The phenol red free serum-free RPMI1640 medium was used. SD± values indicate means of three independent experiments. * $p = 0.05$ and *** $p < 0.001$ are indicated.

Figure 1

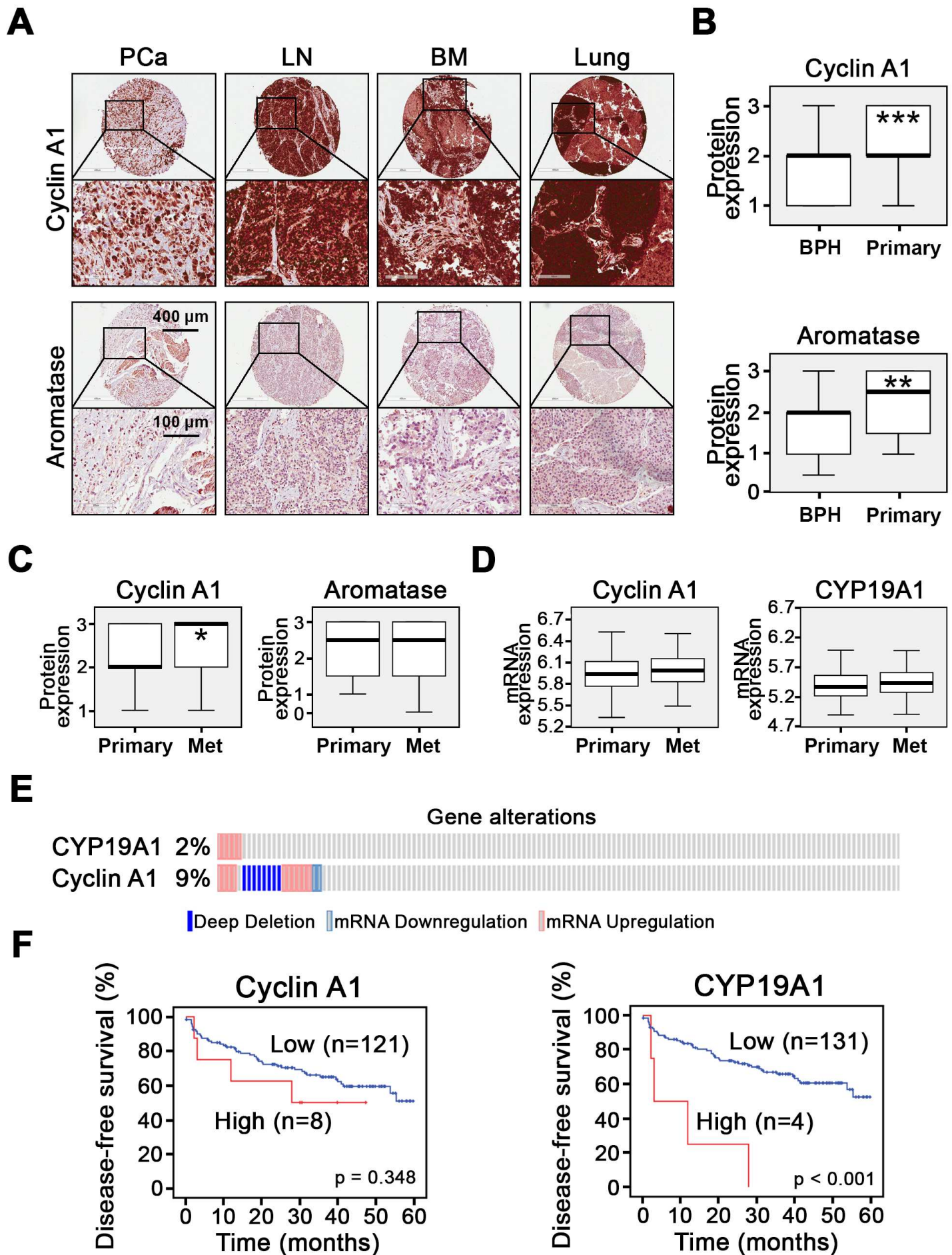


Figure 2

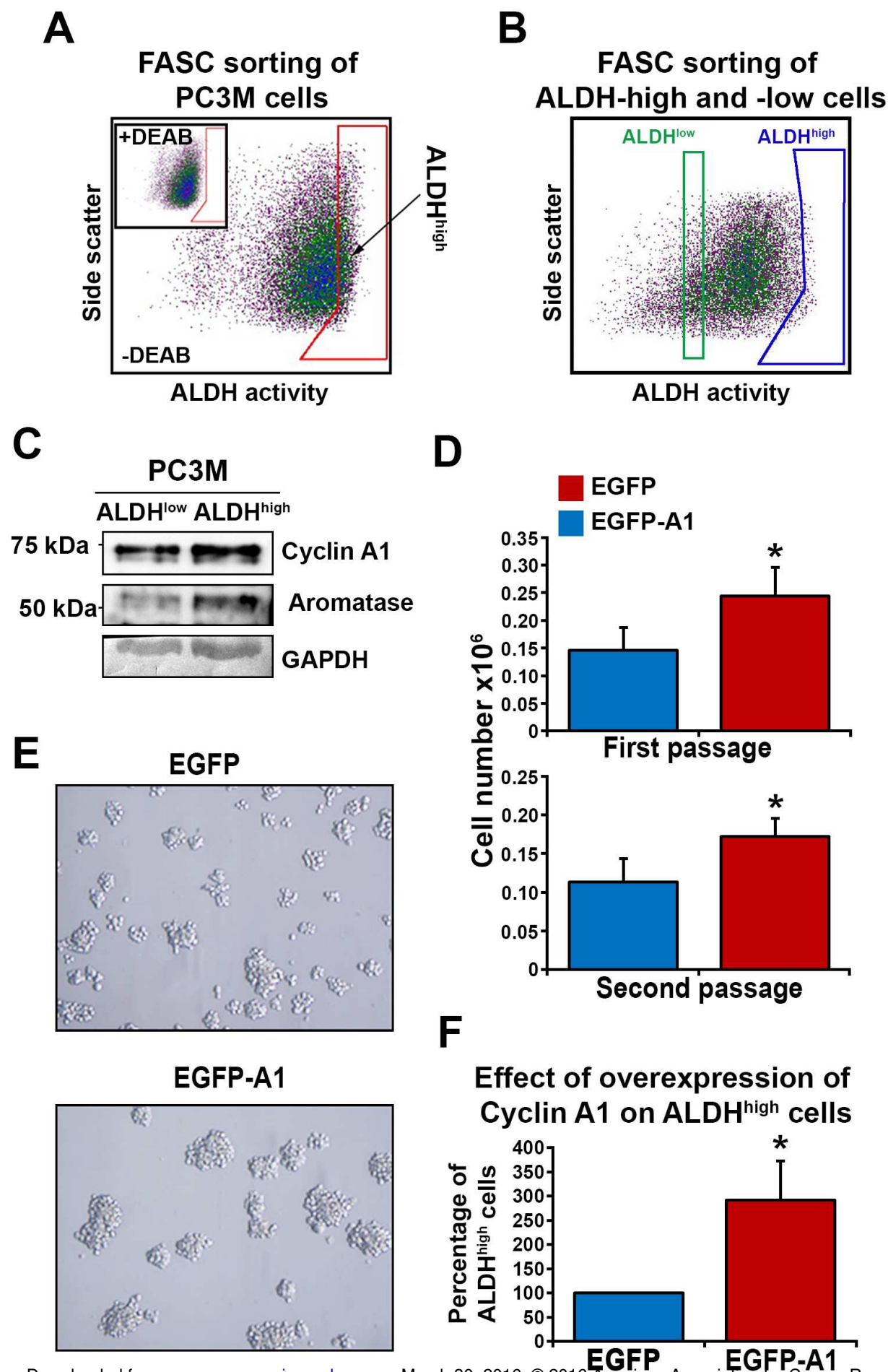
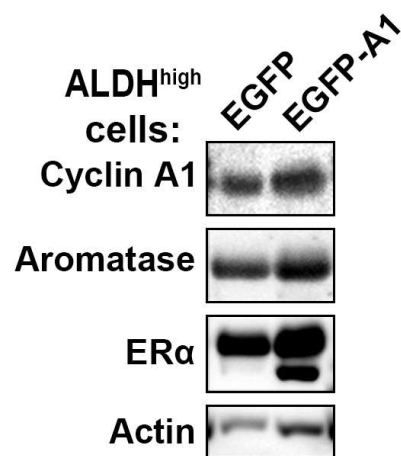
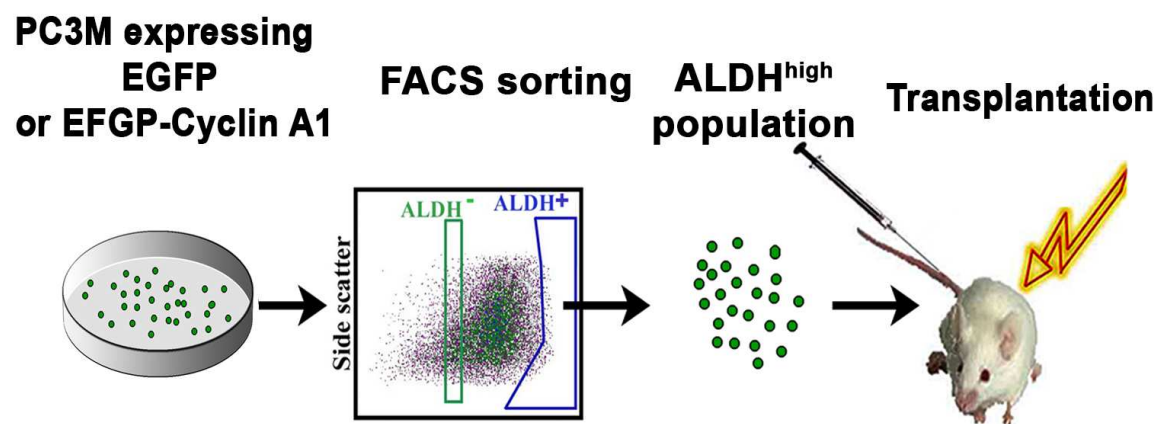


Figure 3

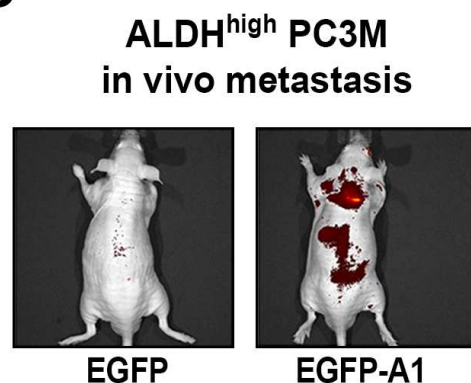
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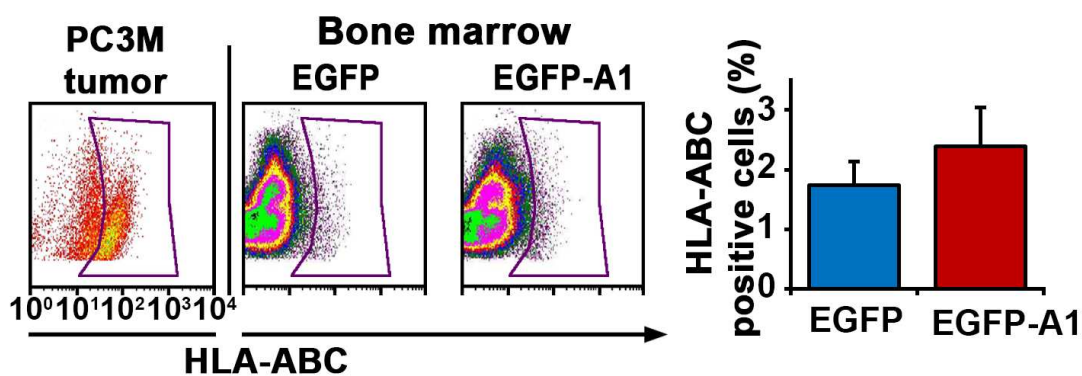
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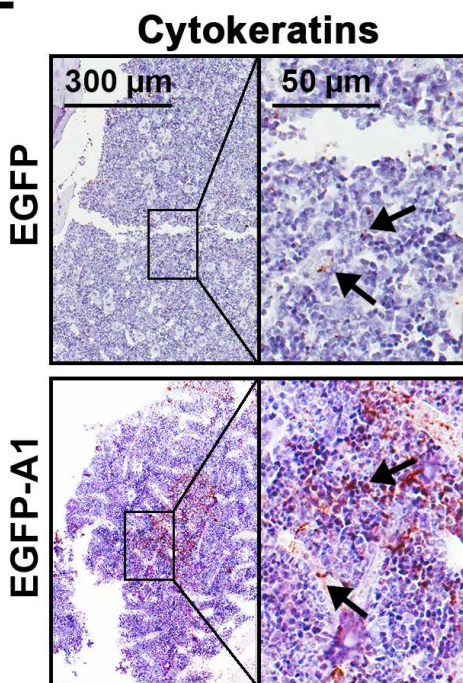
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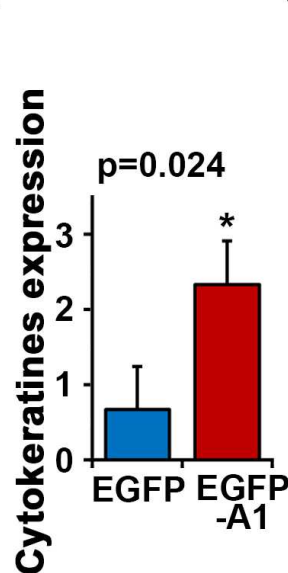
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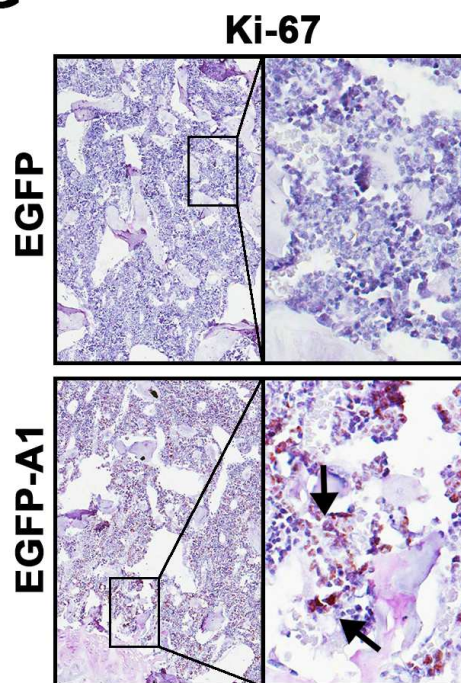
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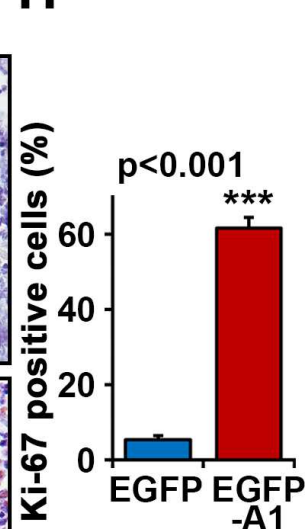
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G



H



Bone marrow

Figure 4

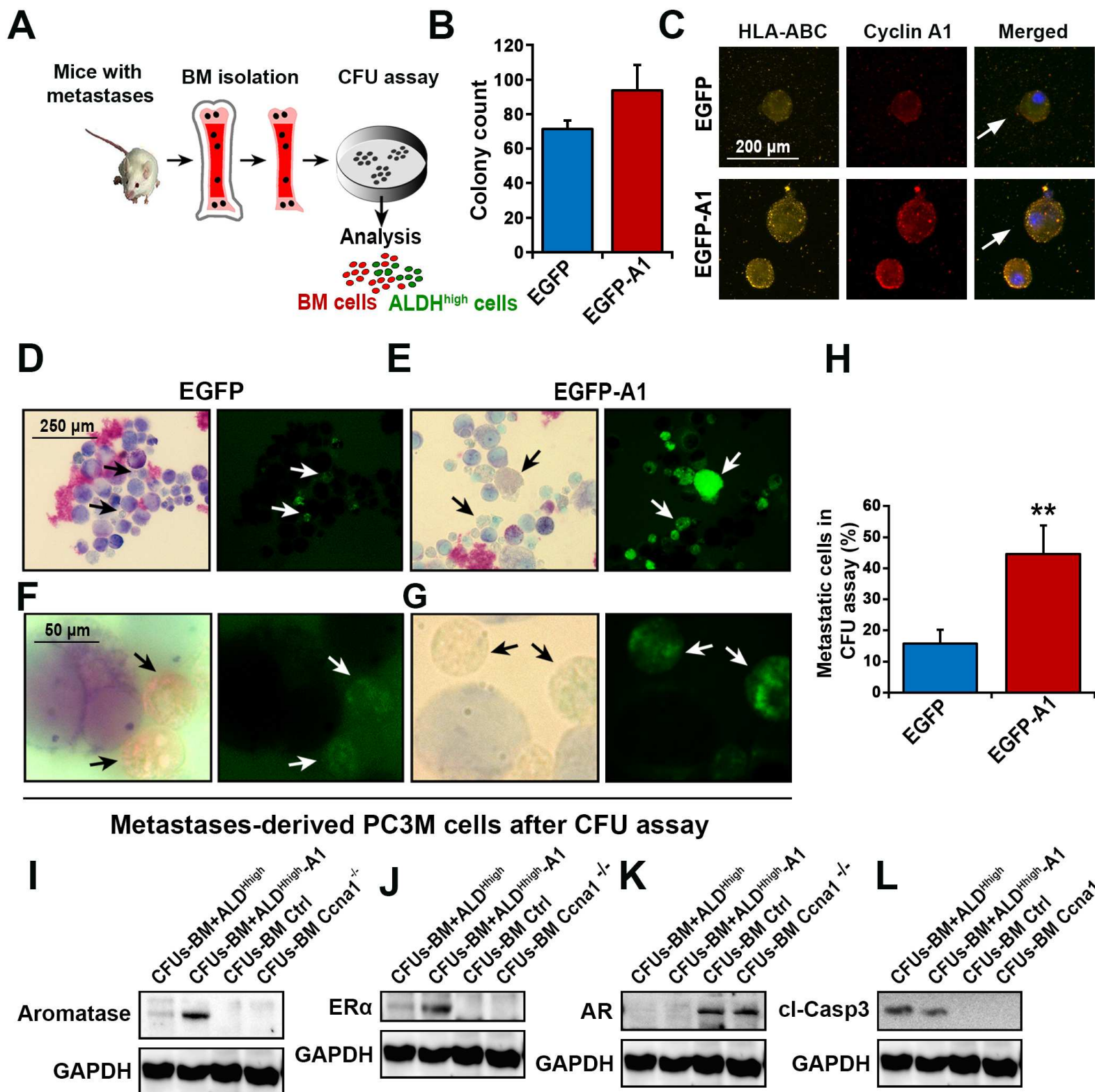
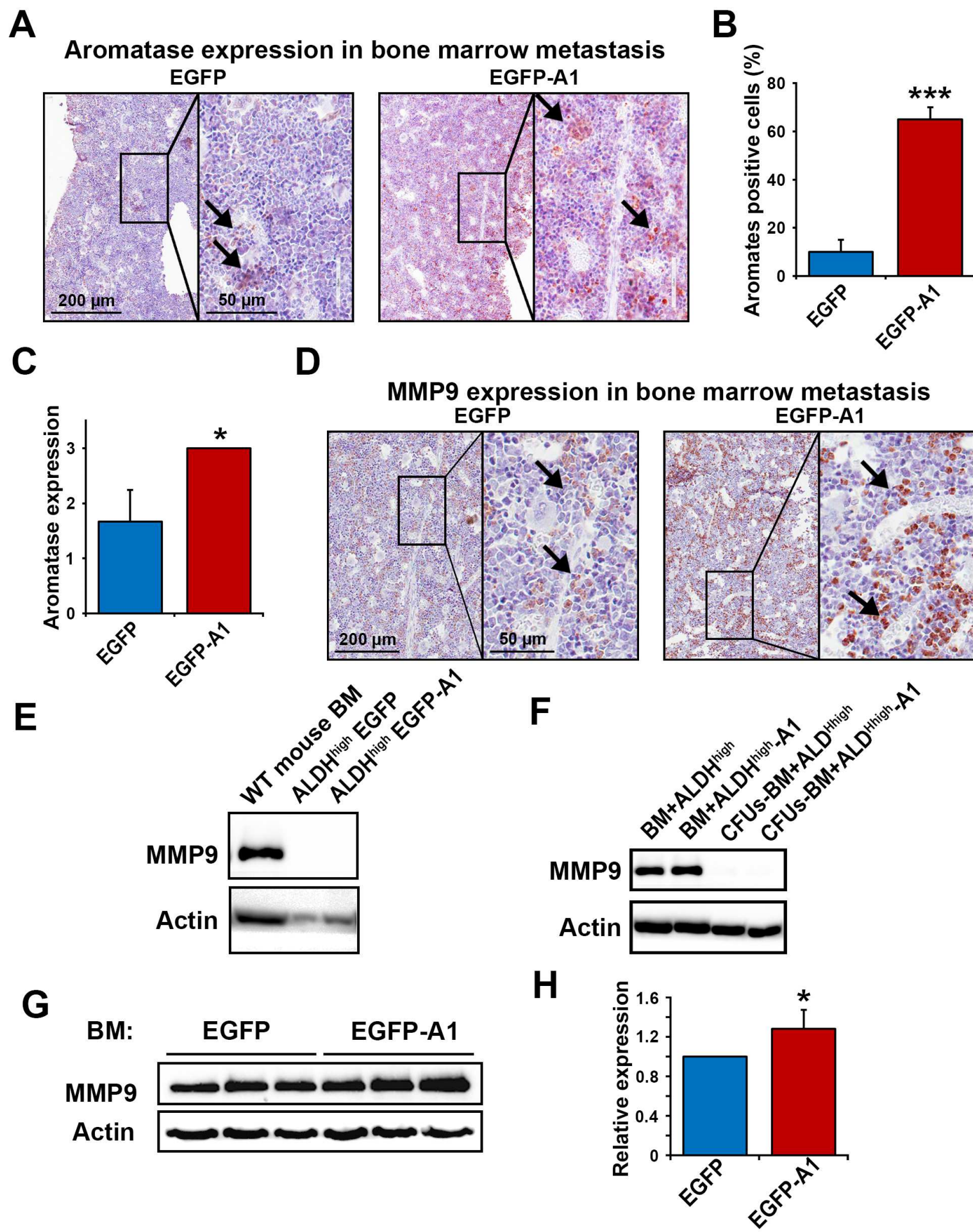
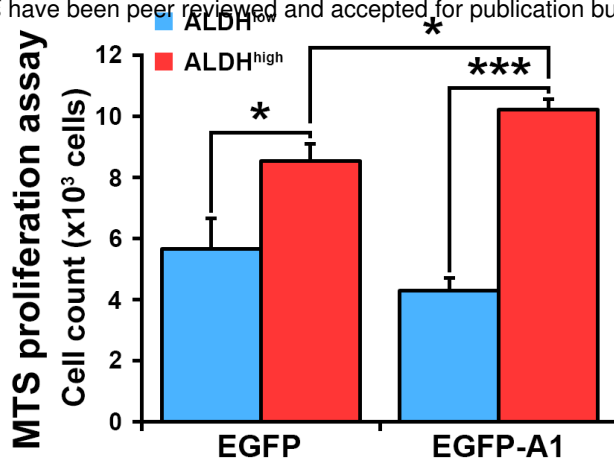


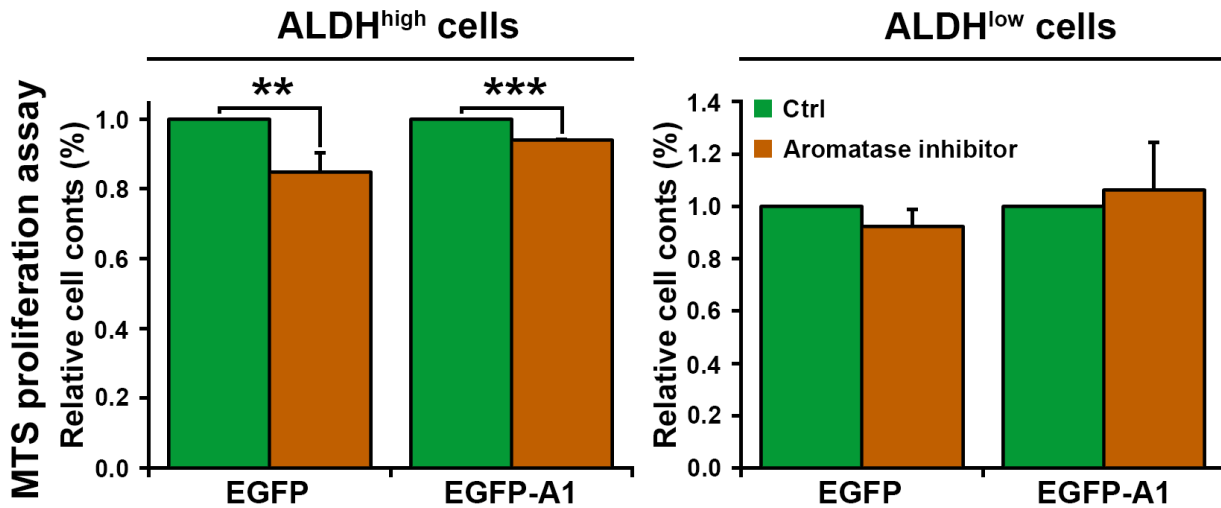
Figure 5





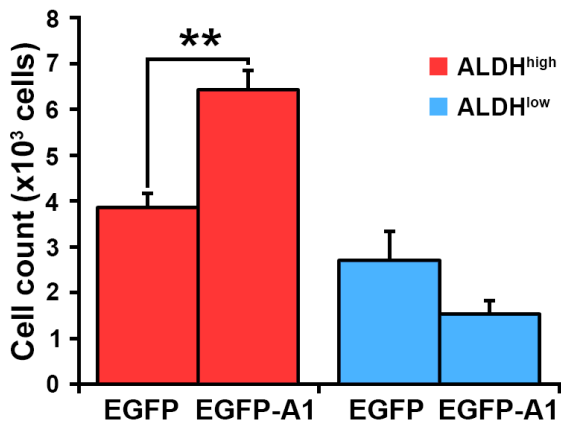
B

Effects of aromatase inhibition



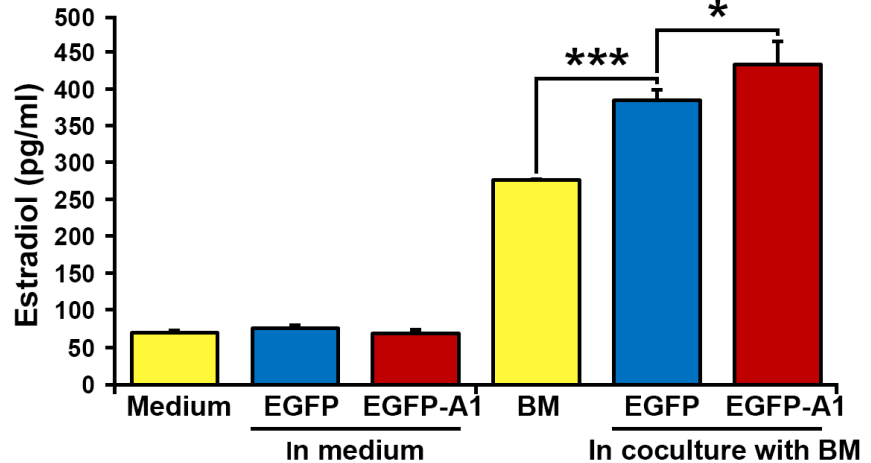
C

Proliferation assay of ALDH^{high} cells cocultured with BM



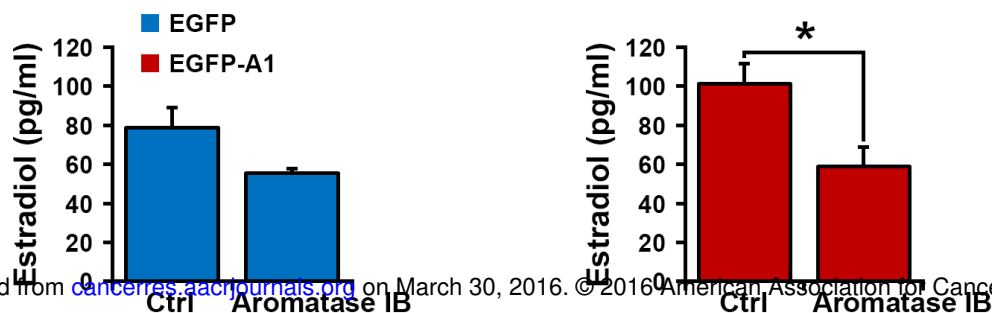
D

Estradiol conversion in ALDH^{high} cells cocultured with BM cells



E

Estradiol in ALDH^{high} cells cocultured with BM cells with or without aromatase inhibitor



Cancer Research

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Cyclin A1 and P450 aromatase promote metastatic homing and growth of stem-like prostate cancer cells in the bone marrow

Regina Miftakhova, Andreas Hedblom, Julius Semenas, et al.

Cancer Res Published OnlineFirst February 26, 2016.

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