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# IL-1β Induces *p62/SQSTM1* and Represses *Androgen Receptor* Expression in Prostate Cancer Cells

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## ABSTRACT

Chronic inflammation is associated with advanced prostate cancer (PCa), although the mechanisms governing inflammation-mediated PCa progression are not fully understood. PCa progresses to an androgen independent phenotype that is incurable. We previously showed that androgen independent, androgen receptor negative ($AR^-$) PCa cell lines have high p62/SQSTM1 levels required for cell survival. We also showed that factors in the HS-5 bone marrow stromal cell (BMSC) conditioned medium can upregulate p62 in $AR^+$ PCa cell lines, leading us to investigate *AR* expression under those growth conditions. In this paper, mRNA, protein, and subcellular analyses reveal that HS-5 BMSC conditioned medium represses *AR* mRNA, protein, and nuclear accumulation in the C4-2 PCa cell line. Using published gene expression data, we identify the inflammatory cytokine, IL-1β, as a candidate BMSC paracrine factor to regulate *AR* expression and find that IL-1β is sufficient to both repress AR and upregulate p62 in multiple PCa cell lines. Immunostaining demonstrates that, while the C4-2 population shows a primarily homogeneous response to factors in HS-5 BMSC conditioned medium, IL-1β elicits a strikingly heterogeneous response; suggesting that there are other regulatory factors in the conditioned medium. Finally, while we observe concomitant AR loss and p62 upregulation in IL-1β-treated C4-2 cells, silencing of *AR* or *p62* suggests that IL-1β regulates their protein accumulation through independent pathways. Taken together, these in vitro results suggest that IL-1β can drive PCa progression in an inflammatory microenvironment through AR repression and p62 induction to promote the development and survival of androgen independent PCa. J. Cell. Biochem. 115: 2188–2197, 2014. © 2014 Wiley Periodicals, Inc.

**KEY WORDS:** INTERLEUKIN-1β; p62/SEQUESTOME-1; ANDROGEN RECEPTOR; PROSTATE CANCER; BONE MARROW STROMAL CELLS; INFLAMMATION

Inflammation is the seventh hallmark of cancer, where pro-inflammatory cytokines activate signaling cascades that promote tumor cell survival, proliferation, angiogenesis, and metastasis [Hanahan and Weinberg, 2011]. In primary and metastatic tumors, bone marrow-derived immune cells can infiltrate the tumor and secrete pro-inflammatory cytokines into the tumor microenvironment [Multhoff et al., 2012]. Interleukin-1 beta (IL-1β) is one such pro-inflammatory cytokine that can support tumor progression through paracrine activation of pro-survival pathways in tumor cells [Multhoff et al., 2012].

IL-1β is produced as a 35 kDa pro-ILβ protein that is cleaved by the inflammasome complex into the 17 kDa biologically active IL-1β protein [Martinon et al., 2002]. Mature IL-1β is then secreted and binds to its heterodimeric IL-1RI/IL-1RAcP receptor on target cells to initiate IL-1β signaling [Dinarello, 2009]. Monocytes are the primary source of IL-1β [Dinarello, 2009]; but IL-1β can also be produced by other cell types such as fibroblasts [Erez et al., 2009] and epithelial cells [Kogan-Sakin et al., 2009] as a paracrine or autocrine signal.

As part of the inflammation response, IL-1β signaling initiates Nuclear Factor Kappa B (NFκB) activation [Lawrence, 2009]. NFκB is a transcription factor that induces expression of pro-inflammatory cytokines, anti-apoptotic proteins, angiogenic proteins, adhesion molecules, extracellular matrix remodeling enzymes, and metastatic factors [Nguyen et al., 2013]. Thus, in the context of chronic inflammation and cancer, IL-1β secreted by bone marrow-derived immune cells can activate NFκB in tumor cells to drive tumor cell proliferation, survival, and metastasis.

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One mechanism of IL-1β activation of NFκB requires the multifunctional adaptor protein, p62 (also known as Sequestome-1) [Sanz et al., 2000; Nakamura et al., 2010]. As an adaptor protein, p62 has several distinct protein–protein interaction domains, two of which are the ubiquitin binding domain and the TRAF6 binding domain [Moscat and Diaz-Meco, 2012]. Tumor necrosis factor receptor associated factor 6 (TRAF6) is an E3 ubiquitin ligase that mediates NFκB activation [Cao et al., 1996; Deng et al., 2000]; and in response to stimuli such as IL-1β and nerve growth factor, p62 binds to and facilitates the polyubiquitination of TRAF6, leading to IκB kinase activation and downstream NFκB nuclear translocation and transactivation [Sanz et al., 2000; Wooten et al., 2005; Nakamura et al., 2010].

Chronic inflammation causes the cytotoxic accumulation of reactive oxygen and nitrogen species, resulting in tissue necrosis and DNA damage that can initiate tumorigenesis [Multhoff et al., 2012]. Once transformed, tumor cells can attenuate intracellular damage from cytotoxic reactive oxygen and nitrogen species through various antioxidant pathways, including activation of the nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor [Mitsuishi et al., 2012].

Under homeostatic conditions, NRF2 is sequestered in the cytoplasm by the adaptor protein, kelch-like ECH-associated protein 1 (KEAP1), marking NRF2 for proteasomal degradation [Mitsuishi et al., 2012]. When ROS is elevated, p62 binds KEAP1 and disrupts the KEAP1–NRF2 interaction, allowing NRF2 to translocate to the nucleus and transactive antioxidant response genes [Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010].

Interestingly, p62 is a gene target for NFκB [Ling et al., 2012] and NRF2 [Jain et al., 2010]. Thus, p62 participates in a positive feedback loop for signaling pathways regulated by both transcription factors. Because NFκB and NRF2 regulated pathways can be antagonistic [Bellezza et al., 2010], p62 is expected to be tightly regulated and function in a dynamic, context-specific manner.

We previously reported that bone marrow stromal cell paracrine factors upregulate p62 in bone metastatic prostate cancer (PCa) cell lines [Chang et al., 2014]. Based on the pleiotropic effects of bone marrow stromal cell paracrine factors on PCa cell lines, including induction of apoptosis, autophagy, or neuroendocrine differentiation [Zhang et al., 2011; Delk and Farach-Carson, 2012], p62 likely has pleiotropic roles in maintaining cellular homeostasis in our model. To begin to gain insight into the role(s) of p62 in PCa cells exposed to bone marrow stromal cell paracrine factors, we first sought candidate paracrine factors that might mediate induction of p62 and chose to explore IL-1β as a candidate cytokine.

IL-1β levels are elevated in tumors [Liu et al., 2013] and serum [Saylor et al., 2012] of prostate cancer (PCa) patients with advance disease. In agreement, IL-1β was shown to induce PCa neuroendocrine differentiation (NED) in vitro [Diaz et al., 1998; Chiao et al., 1999; Albrecht et al., 2004] and promote the skeletal colonization and growth of metastatic PCa cell lines in mice [Liu et al., 2013]. PCa NED is associated with disease progression, poor prognosis, and treatment resistance [Sun et al., 2009]. PCa NED cells produce and secrete proteins that promote tumor cell proliferation, survival, and tumor angiogenesis and do not express the therapeutic target, the androgen receptor (AR) [Sun et al., 2009]. Likewise, PCa bone metastases are aggressive and incurable [Msaouel et al., 2008] and there is evidence that IL-1β accumulation negatively correlates with AR activity and positively correlates with NED in PCa patient bone metastases [Liu et al., 2013].

In this paper, we report that IL-1β can induce *p62* mRNA and repress *AR* mRNA in PCa cell lines and we believe these in vitro results reflect mechanisms by which IL-1β can drive PCa progression and treatment resistance in an inflammatory tumor microenvironment. We propose a model wherein IL-1β, secreted by immune cells in the inflammatory tumor microenvironment or secreted by bone marrow stromal cells in the metastatic niche, can promote the transformation of PCa cells into treatment resistant PCa cells that survive the harsh inflammatory or bone metastatic environments through processes mediated by cell survival proteins like p62.

## MATERIALS AND METHODS

### CELL CULTURE

PCa cell lines (LNCaP, C4-2, MDA PCa 2a) and bone marrow stromal cell lines (HS-5, HS-27a) were grown in a 37°C, 5.0% (v/v) $CO_2$ growth chamber and maintained as described in Chang et al. [2014]. Briefly, LNCaP and C4-2 cell lines were cultured in T-medium (Gibco/Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), MDA PCa 2a cell line was cultured in BRFF-HPC1 medium (0403; AthenaES) supplemented with 20% (v/v) FBS, and HS-5 and HS-27a cell lines were cultured in low glucose DMEM medium (Gibco/Invitrogen) supplemented with 10% FBS.

### CONDITIONED MEDIUM TREATMENT

Bone marrow stromal cell conditioned media was obtained as described in Chang et al. [2014]. Briefly, conditioned T-medium was collected from bone marrow stromal cells after 3 days incubation.

### CYTOKINE AND siRNA TREATMENTS

**Cytokines.** Recombinant human interleukin-1 beta (IL-1β) (201-LB/CF; R&D Systems), recombinant human interleukin-6 (IL-6) (206-IL/CF; R&D Systems). IL-1β was diluted in 0.1% (w/v) filtered bovine serum albumin (BSA) (A7906; Sigma-Aldrich). IL-6 was diluted in 1× sterile phosphate buffered saline (PBS) (71002-822; VWR). 0.1% BSA served as the vehicle control.

**siRNA treatments.** Cells were transfected with a pool of four unique *AR* siRNA duplexes (M-003400-02-0005; Thermo Scientific) or a pool of three unique *p62/SQSTM1* siRNA duplexes (SR305865; Origene) using siTran 1.0 transfection reagent (TT300001; Origene). The Trilencer-27 universal scrambled negative control siRNA duplex was used as a negative control (SR30004; Origene). Immunostaining was used to confirm protein loss.

### WESTERN BLOT ANALYSIS AND ANTIBODIES

Western blot analysis was performed as described in Chang et al. [2014]. Briefly, protein was isolated from cells using NP40 lysis buffer, loaded onto sodium dodecyl sulfate polyacrylamide gel, and transferred from the gel to nitrocellulose membrane. Total protein was visualized using Ponceau S (P7170; Sigma). Protein blot bands were visualized using SuperSignal West Femto chemiluminescent substrate (PI34095; Fisher Scientific) and imaged using the Fujifilm LAS-4000 imager (Fuji). Ponceau S stain or β-actin was used as the loading control.

**Primary antibodies.** Androgen Receptor (AR N-20) (sc-816; Santa Cruz), p62/SQSTM1 (H00008878-M01; Abnova), β-actin (NB600-505; Novus Biologicals).
**Secondary antibodies.** Sheep anti-mouse (515-035-062; Jackson ImmunoResearch Laboratories), goat anti-rabbit (A6154; Sigma-Aldrich).

### RNA EXTRACTION AND QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

RNA was extracted as described in Chang et al. [2014]. Reverse transcription and QPCR were done in a one-step reaction using the Verso 1-step RT-qPCR SYBR Green kit according to the manufacturer's instructions (AB-4104/A; Thermo Scientific). Primers specific for *Androgen Receptor* or *p62/SQSTM1* were used and data was normalized to the β-*actin* transcript levels. Relative mRNA levels were calculated using the$2^{-\Delta\Delta CT}$ method. *Androgen Receptor primers:* Forward: AAG ACG CTT CTA CCA GCT CAC CAA; Reverse: TCC CAG AAA GGA TCT TGG GCA CTT. *p62 primers:* Forward: AAA TGG GTC CAC CAG GAA ACT GGA; Reverse: TCA ACT TCA ATG CCC AGA GGG CTA. β-*actin primers:* Forward: GAT GAG ATT GGC ATG GCT TT; Reverse: CAC CTT CAC CGG TCC AGT TT.

### IMMUNOFLUORESCENCE

Immunofluorescence was carried out as described in Chang et al. [2014]. Briefly, cells were fixed and permeabilized with 100% methanol, blocked with 5% BSA in 1× PBS, and incubated with antibodies at 4°C overnight. Cells were mounted using ProLong Gold Antifade with DAPI (P36935; Life Technologies).
**Primary antibodies.** Androgen receptor (AR N-20) (sc-816; Santa Cruz), p62/SQSTM1 (H00008878-M01; Abnova).
**Fluorescently labeled secondary antibodies.** Alexafluor 488, goat anti-mouse (A11001; Invitrogen), Alexafluor 568, goat anti-rabbit (A11061; Invitrogen).

### MICROSCOPY

Images were taken and processed using the Eclipse TE300 inverted microscope (Nikon) and NIS Elements software (Nikon) or Zeiss axioscope (Carl Zeiss GmbH) and AxioVision software (Carl Zeiss GmbH). Scale bar = 50 μm.

### CELL RESPONSE PERCENTAGE

To determine the percentage of cells in a cell population that down regulated nuclear AR accumulation or enhanced p62 cytoplasmic accumulation, images of immunostained cells were acquired on the Eclipse TE300 inverted microscope (Nikon) and immunostained control-treated cells were used to establish baseline fluorescence intensity for nuclear AR and p62 diffuse cytoplasmic accumulation using NIS Elements software (Nikon). Cells with nuclear AR fluorescence intensity lower than baseline or p62 diffuse cytoplasmic intensity higher than baseline were counted as responsive to treatment. Cells grown in control condition medium, vehicle control, or IL-1β accumulated little to no p62 speckles and were therefore assayed for cytoplasmic p62 intensity. HS-5 BMSC conditioned medium-treated cells were assayed for p62 speckle accumulation. Total cells counts were obtain from five or more microscopy fields for each treatment. Total cell counts for each treatment were as follows: control growth medium, $n > 2{,}300$; HS-5 conditioned medium, $n > 850$; vehicle control, $n > 2{,}300$; IL-1β, $n > 3{,}800$.

### STATISTICS

Statistical significance was determined using unpaired student *t* test.

## RESULTS

### HS-5 BONE MARROW STROMAL CELL PARACRINE FACTORS REPRESS *AR* mRNA, PROTEIN, AND NUCLEAR ACCUMULATION IN C4-2 PCa CELLS

We previously reported that HS-5 BMSC paracrine factor(s) upregulate *p62* mRNA and protein in $AR^+$ PCa cell lines [Chang et al., 2014]. On the other hand, $AR^-$ PCa cell lines have high basal *p62* mRNA [Chang et al., 2014]. This led us to speculate that the loss of AR and the upregulation of p62 accumulation are linked. In accordance, we discovered that HS-5 BMSC conditioned medium down regulated *AR* mRNA, protein, and nuclear accumulation in $AR^+$ C4-2 cells (Fig. 1A–C). However, relative to control growth medium, HS-27a BMSC conditioned medium did not repress *AR* mRNA, protein, or nuclear accumulation in C4-2 cells (Fig. 1A–C). Thus, as with the induction of p62 [Chang et al., 2014], the repression of C4-2 *AR* mRNA, protein, and nuclear accumulation is also specific to the HS-5 BMSC paracrine factor milieu.

### INTERLEUKIN 1-BETA (IL-1β) IS SUFFICIENT TO REPRESS *AR* mRNA AND INDUCE *p62* mRNA IN C4-2 PCa CELLS

The regulation of AR and p62 described above occurs for C4-2 cells grown in HS-5 BMSC conditioned medium, but not for C4-2 cells grown in HS-27a BMSC conditioned medium [Fig. 1; Chang et al., 2014]. Therefore, we sought candidate paracrine factor(s) that might mediate HS-5 BMSC regulation of AR and p62 in C4-2 cells. Comparative gene expression analysis of HS-5 BMSCs versus HS-27a BMSCs led us to test the effect of interleukin-1 beta (IL-1β) on AR and p62 accumulation in C4-2 cells. *IL-1*β mRNA levels are approximately 83 times higher in HS-5 BMSCs than HS-27a BMSCs [Graf et al., 2002] and HS-5 BMSCs secrete IL-1β protein [Roecklein and Torok-Storb, 1995]. Furthermore, IL-1β has been shown to block AR activity in a PCa cell line [Culig et al., 1998]. Indeed, we found that 25 ng/ml IL-1β was sufficient to represses *AR* mRNA levels and nuclear accumulation in C4-2 cells (Figs. 2A and B). In addition, IL-1β upregulated *p62* mRNA levels and protein accumulation in C4-2 cells (Figs. 2A and B).

p62 sequesters ubiquitinated protein aggregates into the autophagosome for degradation, therefore, p62 accumulation could indicate aberrant autophagy-mediated degradation of p62 [Bjorkoy et al., 2005]. Treatment with the autolysosomal inhibitor, chloroquine, increased p62 accumulation in IL-1β-treated cells, demonstrating that autophagy-mediated degradation of p62 was preserved in IL-1β-treated cells (Fig. 2C). Thus, as with HS-5 BMSC conditioned medium, IL-1β can repress AR and upregulate p62.

### THE C4-2 PCa CELL POPULATION SHOWS A HETEROGENEOUS RESPONSE TO IL-1β REGULATION OF AR AND p62

While, both HS-5 BMSC conditioned medium and IL-1β can repress *AR* mRNA and induce *p62* mRNA in C4-2 cells [Figs. 1 and 2; Chang et al., 2014], IL-1β is likely not the only factor in the HS-5 BMSC

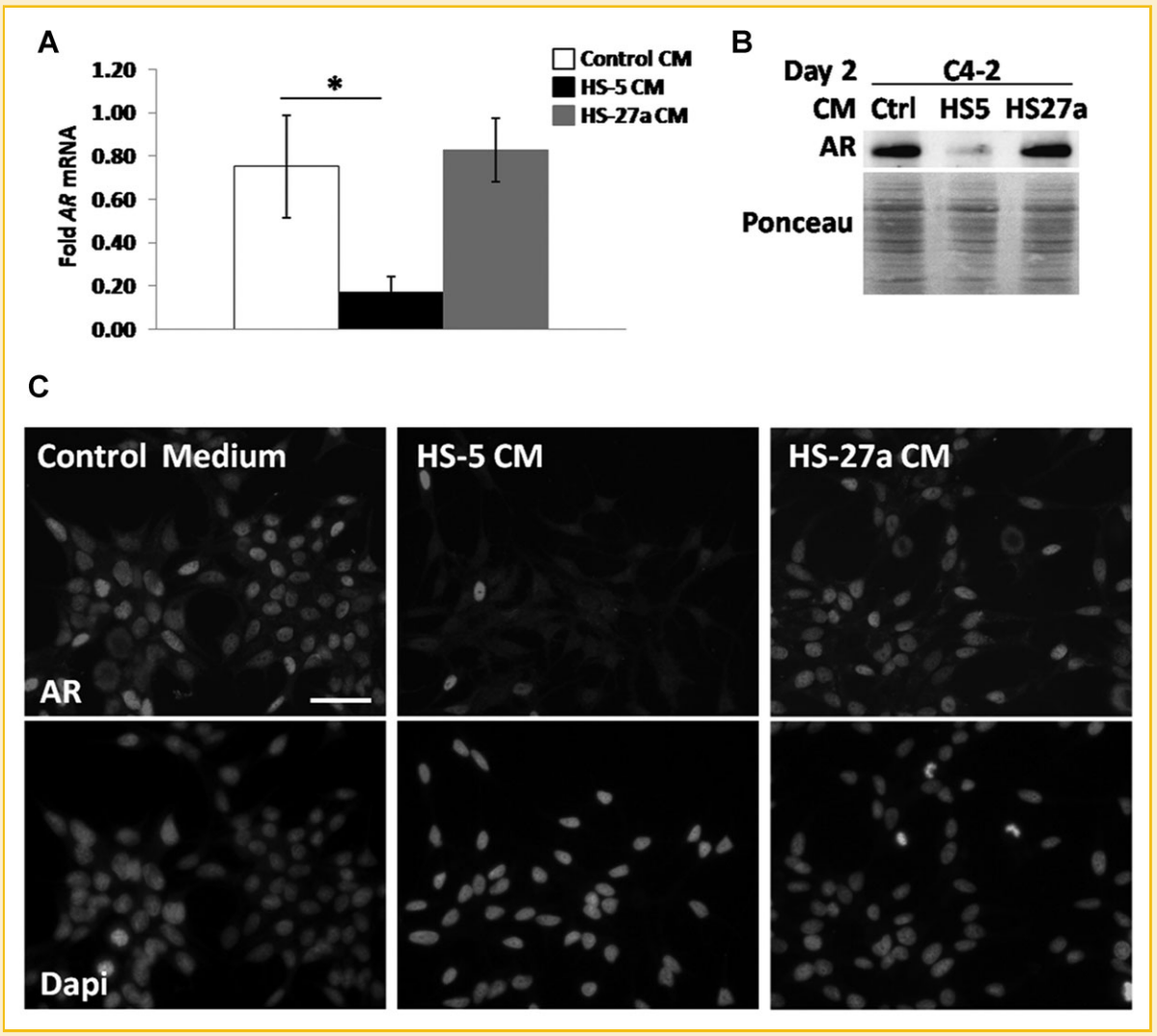


**Fig. 1.** HS-5 BMSC conditioned medium represses AR in C4-2 PCa cells. C4-2 cells were grown in control growth medium or conditioned medium from HS-5 or HS-27a BMSCs for 2 days. C4-2 cells were analyzed for *AR* mRNA, protein, or subcellular localization. A: QPCR, (B) Western blot, and (C) immunostaining for AR revealed that, in comparison to control or HS-27 BMSC conditioned media, HS-5 BMSC conditioned medium repressed *AR* mRNA, protein, and nuclear accumulation in C4-2 cells. For QPRC, relative mRNA levels were normalized to control growth medium; error bars represent standard deviation of three biological replicates; **P*-value = 0.008. For Western blot, Ponceau stain serves as a loading control. For immunostaining, dapi is a nuclear stain and scale bar = 50 μm.

milieu regulating these proteins. For example, HS-5 BMSC conditioned medium reproducibly enhanced p62 speckle accumulation in C4-2 cells (Fig. 3, data not shown), while treatment with 25 ng/ml IL-1β for a similar period of time primarily enhanced diffuse p62 accumulation (Figs. 2B and 3, data not shown). p62 speckles or aggregates are the active organizing centers for various p62-mediated signaling pathways [Moscat and Diaz-Meco, 2009]. This suggests that while both factors in HS-5 BMSC conditioned media and IL-1β can induce *p62* mRNA, their posttranslational regulation of p62 may differ.

In preliminary experiments, various recombinant human IL-1β concentrations and treatment time points could induce *p62* mRNA or protein accumulation in multiple cell lines, including the LNCaP, C4-2, and MDA PCa 2A prostate cancer cell lines and the T47D breast cancer cell line (Supplementary Fig. S1; data not shown). However, the level of p62 induction observed in pooled cell populations using QPCR or Western blot analysis was either subtle or inconsistently detectable (data not shown). Therefore, we employed a more sensitive and informative approach by using immunostaining to analyze the effects of IL-1β on individual cells. Focusing on the C4-2 cell line, we co-immunostained for AR and p62 and discovered that, while the C4-2 cell population showed a largely uniform response to factors in HS-5 BMSC conditioned medium, only a portion of the cells responded to treatment with recombinant human IL-1β (Figs. 3 and 4). For example, we found that 93% of the C4-2 cell population grown in HS-5 BMSC conditioned medium for 2 days showed reduced or no nuclear AR staining, 100% showed p62 speckle accumulation, and 93% of the population showed concomitant loss of nuclear AR and enhanced p62 speckle accumulation (Fig. 4). On the other hand, 66% of the C4-2 cell population grown in 25 ng/ml

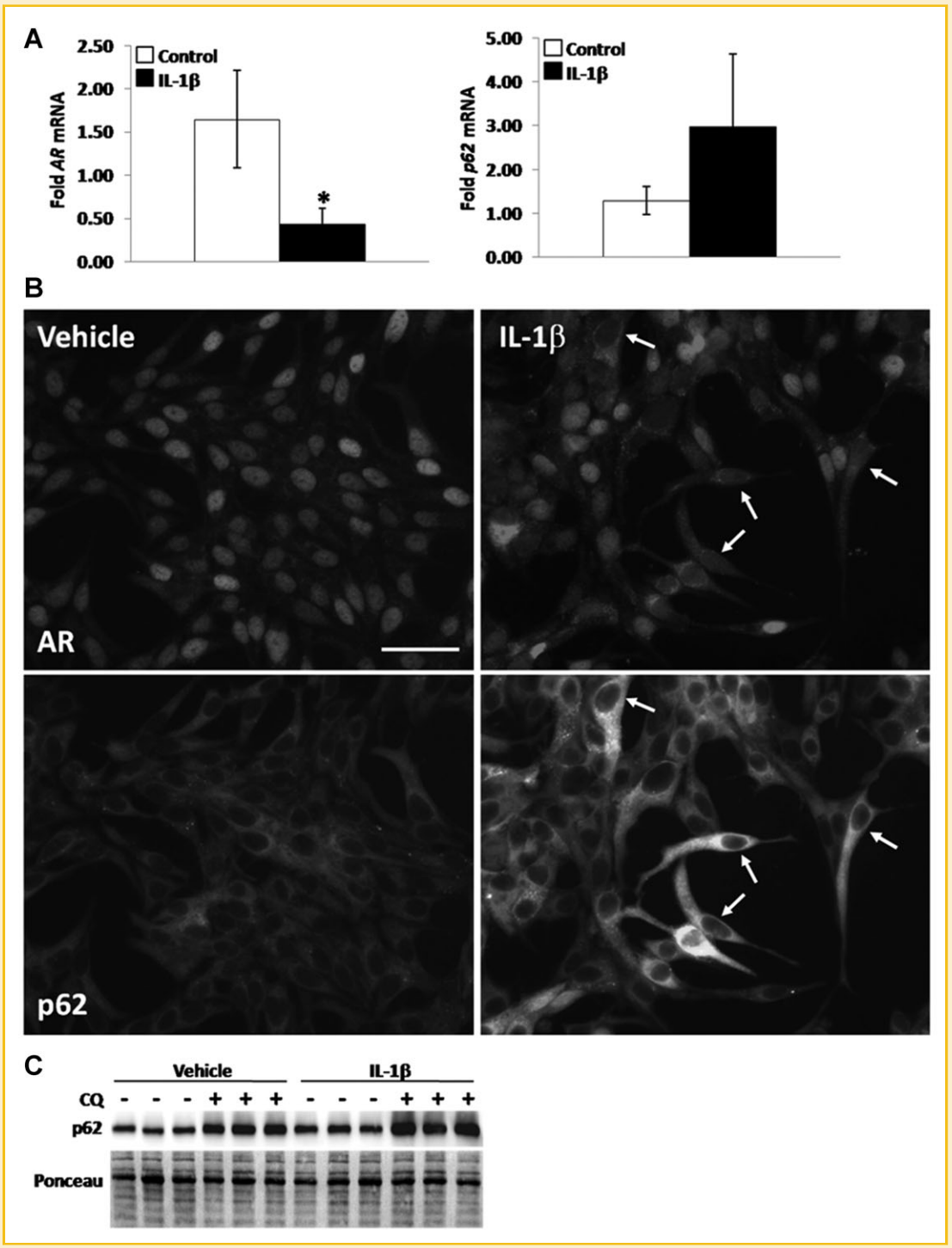


Fig. 2. IL-1β represses AR and induces p62 in C4-2 PCa cells. C4-2 cells were grown in vehicle control or 25 ng/ml recombinant human IL-1β protein for 2 days and analyzed for AR or p62 accumulation. A: QPCR demonstrated that IL-1β was sufficient to repress *AR* mRNA accumulation (*P*-value = 0.01) and upregulate *p62* mRNA levels (*P*-value = 0.08) in C4-2 cells. Relative mRNA levels were normalized to control growth medium and error bars represent standard deviation of three biological replicates. B: Co-immunostaining for AR and p62 protein revealed that IL-1β repressed nuclear accumulation and induced *p62* subcellular protein accumulation in C4-2 cells. Scale bar = 50 μm. C: C4-2 cells were grown for 3 days in vehicle control or 25 ng/ml recombinant human IL-1β, in the absence or presence of 40 μM chloroquine (CQ), and analyzed for p62 protein by Western blot. CQ blocks autophagy-mediated degradation of p62 in the vehicle control and in the presence of IL-1β, indicating that IL-1β induction of p62 is not due to abrogated p62 degradation.

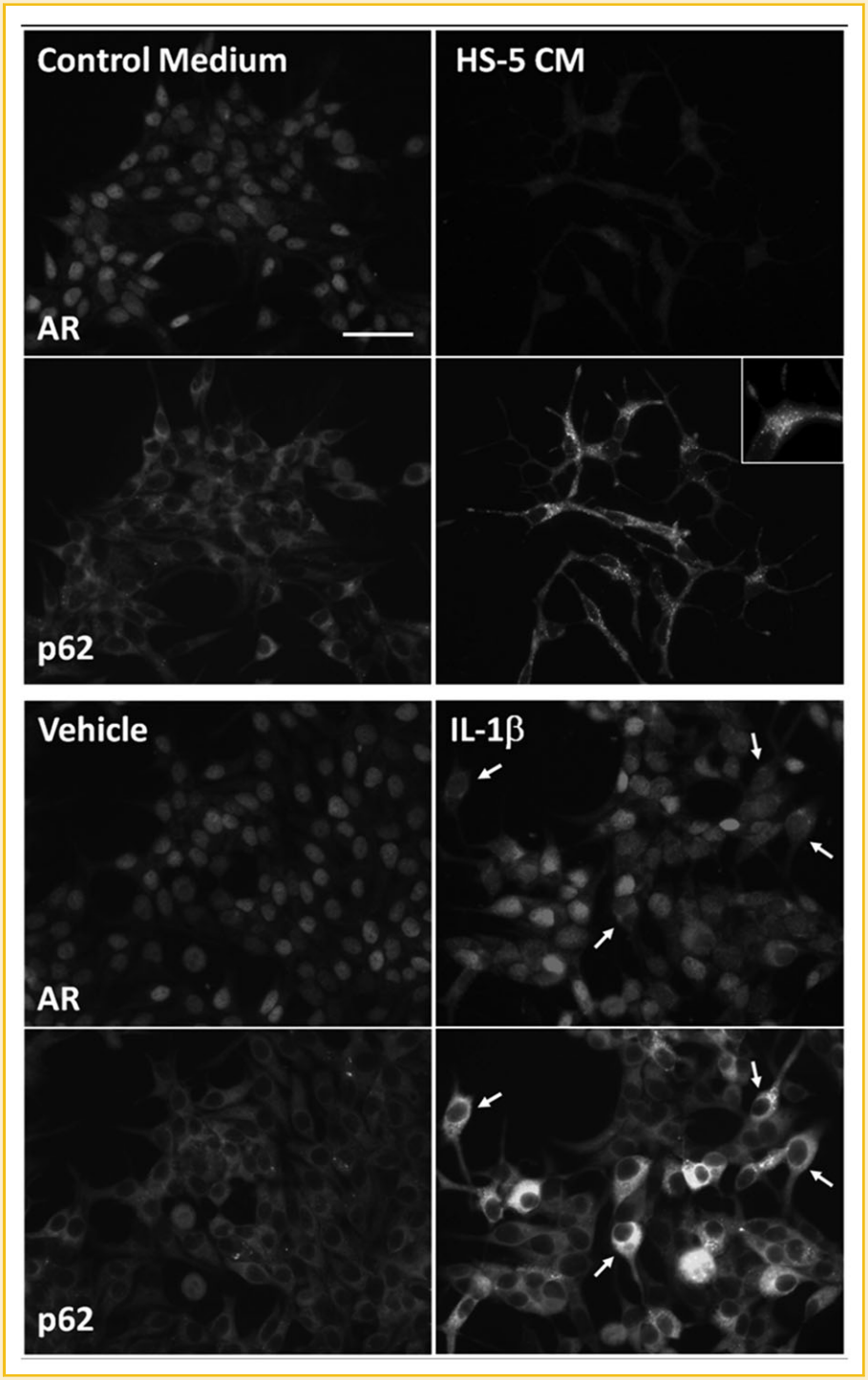


**Fig. 3.** HS-5 BMSC conditioned medium and IL-1β differently regulate p62 subcellular accumulation. C4-2 cells were grown in HS-5 BMSC conditioned medium or 25 ng/ml recombinant human IL-1β protein for 2 days and co-immunostained for AR and p62. HS-5 BMSC conditioned medium repressed AR nuclear accumulation and induced p62 speckle accumulation (inset). IL-1β repressed AR nuclear accumulation and induced diffuse p62 accumulation (representative cells indicated by arrows). Scale bar = 50 μm.

IL-1β for 2 days showed AR loss, 18% showed p62 induction, and 9% showed concomitant AR loss and p62 induction (Fig. 4). Thus, the response of the C4-2 cell population to HS-5 BMSC conditioned medium regulation of AR and p62 is primarily homogenous, while the response to IL-1β alone is strikingly heterogeneous.

The heterogeneous response of the C4-2 PCa cell population to IL-1β could not be attributed to treatment with insufficient recombinant IL-1β protein concentration; for, while our Western blot method could detect the 17 kDa IL-1β protein in 20 μl of 25 ng/ml of recombinant IL-1β protein, we were not able to detect IL-1β

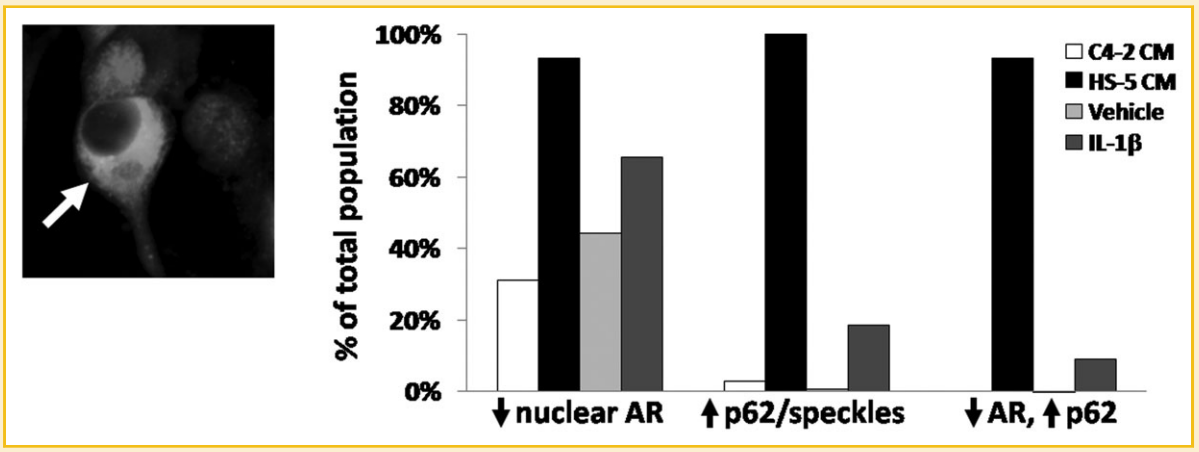


Fig. 4. The C4-2 PCa cell population shows a heterogeneous response to IL-1β regulation of AR and p62. C4-2 cells were grown in HS-5 BMSC conditioned medium or 25 ng/ml recombinant human IL-1β protein for 2 days and co-immunostained for AR (red) and p62 (green). *Image:* Shown is a merged image of a representative responsive cell showing concomitant nuclear AR loss and diffuse cytoplasmic p62 upregulation (arrow). *Graph:* The percentage of cells showing the loss of nuclear AR, the upregulation of speckled/diffuse p62, or both was graphed. Ninety-three percent of C4-2 cells grown in HS-5 BMSC conditioned medium showed concomitant loss of nuclear AR and induction of p62 speckles, while 9% of the C4-2 cells grown in IL-1β showed concomitant AR loss and diffuse p62 induction, indicating that the response of the C4-2 cell population to IL-1β is heterogeneous.

protein in a comparable volume of the HS-5 BMSC conditioned medium (Supplementary Fig. S1). Thus, factors other than, or in addition to, IL-1β in the HS-5 BMSC conditioned medium regulate *AR* and *p62* mRNA and protein levels in C4-2 PCa cells.

### IL-1β REPRESSES AR AND UPREGULATES p62 THROUGH INDEPENDENT PATHWAYS

Given that factors in HS-5 conditioned medium and IL-1β can both regulate AR and p62 in C4-2 cells (Figs. 1–4) and given that $AR^-$ PCa cell lines have high basal p62 levels [Chang et al., 2014], we hypothesized that AR and p62 regulation are interdependent. To test our hypothesis, we used siRNA to down-regulate *AR* mRNA and protein in $AR^+$ C4-2 PCa cells and co-immunostained cells for AR and p62 to determine the effect on p62 accumulation. Under control growth conditions, loss of AR did not upregulate p62 accumulation (Fig. 5). Likewise, loss of AR protein did not enhance IL-1β upregulation of p62 in C4-2 PCa cells (Fig. 5). Conversely, siRNA-mediated loss of p62 protein in C4-2 PCa cells did not prevent the down regulation of AR induced by IL-1β (Fig. 5). Furthermore, analysis of the heterogeneous response of C4-2 cells to IL-1β, revealed that of the 66% of cells that showed reduced nuclear AR accumulation, 57% lost nuclear AR without a concomitant increase in p62; and of the 18% of cells that increased p62 accumulation, 9% upregulated p62 without the loss of nuclear AR (Fig. 4). Taken together, these data suggest that the IL-1β regulation of p62 induction and AR repression are independent pathways.

## DISCUSSION

### IL-1β CAN CONTRIBUTE TO ANDROGEN-INDEPENDENT PCa BY REPRESSING *AR* EXPRESSION AND UPREGULATING p62

Chronic inflammation is implicated in PCa progression [Gueron et al., 2012]. One course of PCa progression is the development of androgen independent PCa, which is often metastatic and is incurable [Grossmann et al., 2001]. Androgen independent PCa cells have reduced or no dependence on androgen for survival due to AR over-accumulation or AR gain-of-function mutations [Grossmann et al., 2001]. Androgen independences can also result from loss of *AR* expression and the upregulation of compensatory cell survival mechanism (e.g., Bcl-2 overexpression) [Grossmann et al., 2001; Sun et al., 2009]. As such, chronic inflammation likely promotes PCa progression to androgen-independent disease through inflammatory cytokine signaling. As demonstrated in this report, the inflammatory cytokine, IL-1β, can repress *AR* expression in PCa cell lines (Fig. 2 and Supplementary Fig. S1). Thus, we propose that IL-1β secreted by bone marrow-derived immune cells infiltrating the primary PCa tumor or secreted by bone marrow stromal cells in the PCa bone metastatic niche, can repress *AR* expression, thereby contributing to androgen independence.

Because the loss of AR can be cytotoxic for PCa cells, $AR^-$ PCa subtypes (e.g., small cell PCa, neuroendocrine PCa) have high levels of pro-survival proteins [Sun et al., 2009]. Indeed, we have shown that the $AR^-$ PCa cell lines, DU145 and PC3, have high basal p62 levels that is required for their cell survival [Chang et al., 2014]. In this report, we have shown that IL-1β upregulates *p62* expression in PCa cell lines (Fig. 2 and Supplementary Fig. S1). Therefore, it is intriguing to speculate that chronic exposure to IL-1β in an inflammatory tumor microenvironment contributes to androgen independence, not only by repressing PCa *AR* expression, but also by upregulating p62 to help maintain cellular homeostasis.

According to the GeneCard database, p62 forms various complexes with at least 30 different proteins. Therefore, p62 could be involved in a myriad of processes that protect PCa cells in an inflammatory environment. For example, it is plausible that IL-1β-induced *p62* expression increases the pool of p62 available to integrate cues from the NFκB inflammatory response, NRF2 antioxidant response, and autophagy, in order to attenuate cytotoxic ROS accumulation and

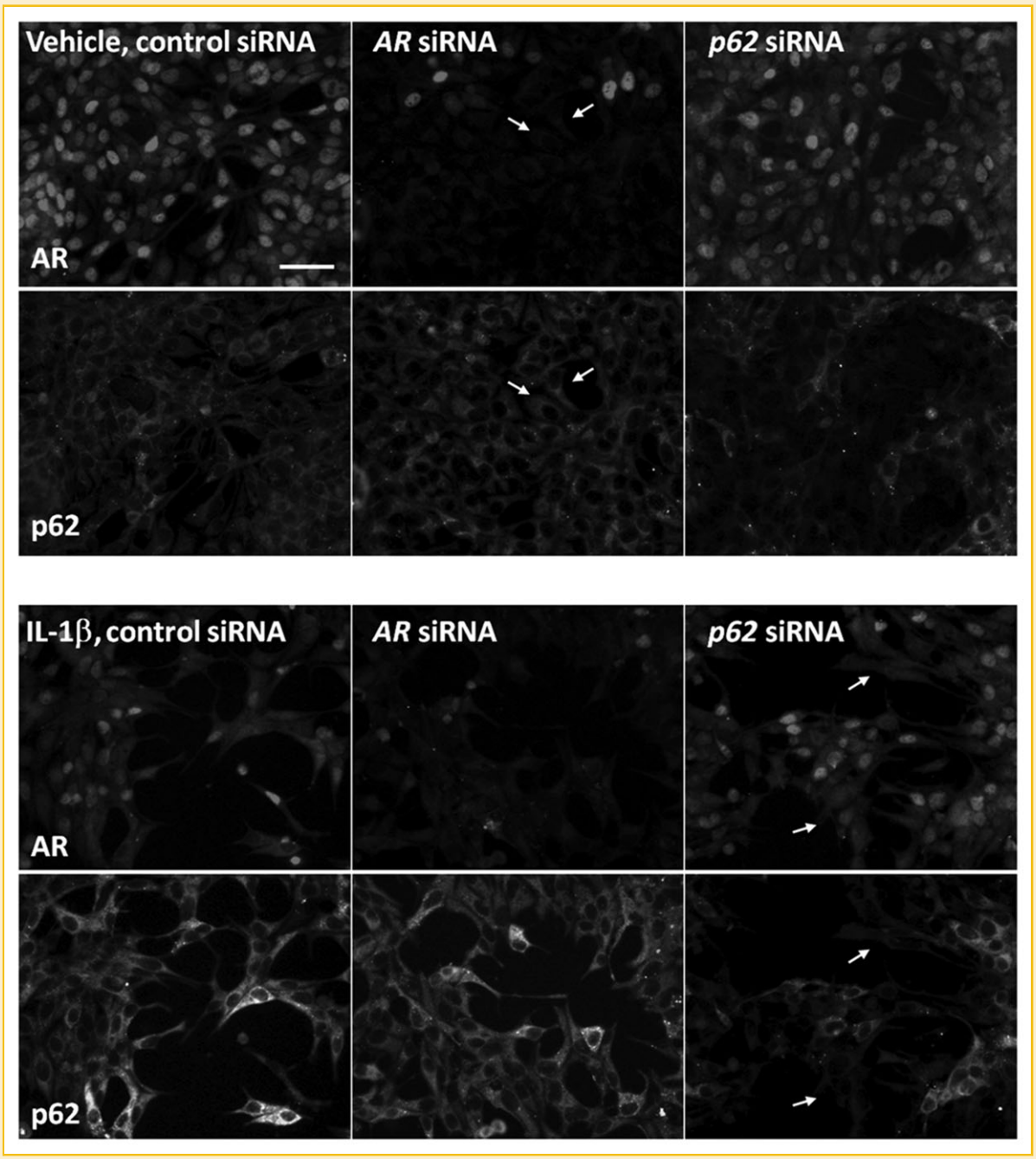


**Fig. 5.** IL-1β regulates AR and p62 through independent pathways. C4-2 cells were grown vehicle control or 25 ng/ml recombinant human IL-1β protein for 2 days in the presence of 40 μM control siRNA, *AR* siRNA, or *p62* siRNA and then co-immunostained for AR and p62. Loss of AR protein did not induce p62 accumulation in vehicle control-treated or IL-1β-treated cells. Loss of p62 protein did not down regulate AR in vehicle control-treated cells or inhibit IL-1β-mediated AR repression. Arrows indicate representative cells.

clear ROS-damaged protein aggregates. It will be important to identify the role(s) of p62 in PCa cell response to IL-1β to elucidate potential mechanism of PCa disease progression.

One aspect of PCa progression is development of treatment resistance; and androgen independence is characteristic of treatment-resistant, metastatic disease. For example, the AR-targeting therapies, androgen deprivation therapy (ADT) and anti-androgens are initially effective at attenuating PCa progression. However, many patients subsequently develop androgen independent PCa cell growth and relapse within a few years of treatment [Beltran et al., 2011]. Cytotoxic chemotherapy is employed as a first line of defense against androgen independent PCa, but has historically shown limited efficacy [Beltran et al., 2011]. Our data suggest that IL-1β could contribute to such therapeutic resistance by repressing expression of the therapeutic target, *AR*, thereby rendering ADT or anti-androgens ineffective. Furthermore, by upregulating pro-

survival p62, IL-1β could protect PCa cells from cytotoxic chemotherapy. Despite extensive clinical studies of various drugs, including chemotherapeutic agents, radiopharmaceuticals, and bisphosphonates, androgen independent PCa, particularly bone metastatic PCa, remains incurable [Beltran et al., 2011; Mukherji et al., 2014]. Therefore, based on our data, we contend that IL-1β-targeted therapy should be explored as an adjuvant to both mitigate and treat androgen independent PCa. Ongoing clinical trials using an IL-1 receptor antagonist in combination with chemotherapy for various advanced cancers (ClinicalTrials.gov) will provide useful insight into this treatment strategy.

### FACTORS OTHER THAN IL-1β LIKELY MEDIATE HS-5 BMSC REGULATION OF AR AND p62 IN PCa CELLS

Among its pleiotropic effects on PCa cells, HS-5 BMSC conditioned media induces neuroendocrine differentiation [Zhang et al., 2011], induces cytoprotective autophagy [Delk and Farach-Carson, 2012], represses *AR* expression (Fig. 1), and upregulates *p62* expression and speckle accumulation [Fig. 3; Chang et al., 2014]. Taken together, the HS-5 BMSC conditioned medium paracrine factors promote the development and maintenance of androgen independent PCa by altering molecular programs (e.g., loss of *AR* expression) and capitalizing on cytoprotective mechanisms (e.g., autophagy induction); and identifying these paracrine factors will be important for developing therapeutic interventions.

Using the results of differential gene expression analysis [Graf et al., 2002], we focused on IL-1β and, previously, on IL-6, as candidate paracrine factors because their genes are highly expressed in HS-5 BMSCs, they are secreted by HS-5 BMSCs [Roecklein and Torok-Storb, 1995], and both IL-1β and IL-6 can induce PCa neuroendocrine differentiation in vitro [Diaz et al., 1998; Sun et al., 2009]. We previously reported that IL-6 induces autophagy [Delk and Farach-Carson, 2012] and in this report, we demonstrate that IL-1β both represses *AR* and induces *p62* expression (Fig. 2 and Supplementary Fig. S1) in PCa cells. Unlike IL-6, which is readily detectable by Western blot in HS-5 BMSC conditioned medium [Delk and Farach-Carson, 2012], under the same conditions, we do not detect IL-1β in the HS-5 BMSC conditioned by Western blot, but can detect recombinant human IL-1β (Supplementary Fig. S1). In addition, the recombinant human IL-1β concentrations we used in our experiments elicit only a heterogeneous response for AR and p62 regulation in the PCa cell population, while HS-5 BMSC conditioned media elicits a nearly homogeneous response (Figs. 3 and 4). Thus factors other than, or in addition to, IL-1β mediate HS-5 BMSC repression of *AR* and upregulation of *p62* expression in PCa cells.

Finally, in addition to upregulating *p62* expression, factors in HS-5 BMSC conditioned medium promote p62 speckle accumulation in PCa cells (Fig. 3). However, treatment with IL-1β for the same period of time does not induce p62 speckles (Fig. 3). Thus, paracrine factors other than IL-1β are likely signaling the formation of active p62 complexes in PCa cells exposed to HS-5 BMSC conditioned medium.

### THE HETEROGENEOUS RESPONSE OF THE C4-2 PCa CELL LINE TO IL-1β IS A USEFUL MODEL FOR TUMOR HETEROGENEITY

While virtually the entire C4-2 cell population shows concomitant AR loss and p62 upregulation when grown in HS-5 BMSC conditioned medium, it is unclear why only a small percentage of the C4-2 cell population shows the same response when grown in a sufficient concentration of IL-1β for the same time period (Figs. 3 and 4). We cannot attribute the heterogeneous response of the C4-2 cells to degraded, inactive recombinant human IL-1β, because full length IL-1β is remains detectable in the media by Western blot throughout the duration of the experiment (data not shown). In addition, the cells are grown in a monolayer in vitro, allowing equal exposure to the recombinant human IL-1β. The strikingly heterogeneous response suggests that there is a subpopulation of IL-1β sensitive cells in the otherwise isogenic cell line that may ultimately have a survival advantage by upregulating proteins like p62 or by developing into androgen independent cells through the loss of *AR* expression. Importantly, we can take advantage of the heterogeneous response to IL-1β and use systems biology approaches to identify relevant molecular networks that can predict those cells in a heterogeneous tumor that will develop into aggressive disease as a result of chronic inflammation and predict cell populations that would respond to cytokine-targeted therapy.

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