NRH:quinone Oxidoreductase 2 (NqO2) and Glutaminase (GLS) Both Play a Role in Large Extracellular Vesicles (LEV) Formation in Preclinical LNCaP-C4-2B Prostate Cancer Model of Progressive Metastasis

Thambi Dorai  
*New York Medical College*

Ankeeta Shah

Faith Summers

Rajamma Mathew  
*New York Medical College*

Jing Huang  
*New York Medical College*

*See next page for additional authors*

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Authors
Thambi Dorai, Ankeeta Shah, Faith Summers, Rajamma Mathew, Jing Huang, Tze-Chen Hsieh, and Joseph M. Wu

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NRH:Quinone Oxidoreductase 2 (NQO2) and Glutaminase (GLS) both play a role in large extracellular vesicles (LEV) formation in preclinical LNCaP-C4-2B prostate cancer model of progressive metastasis.

Thambi Dorai1,2, Ankeeta Shah3, Faith Summers2, Rajamma Mathew4,5, Jing Huang4, Tze-chen Hsieh2, Joseph M.Wu2

1. Department of Urology, New York Medical College, Valhalla, NY 10595
2. Department of Biochemistry & Molecular Biology, New York Medical College, Valhalla, NY 10595
3. Committee on Genetics, Genomics and Systems Biology, University of Chicago, Chicago IL 60637
4. Section of Pediatric Cardiology, Department of Pediatrics, New York Medical College, Valhalla, NY 10595.
5. Department of Physiology, New York Medical College, Valhalla, NY 10595

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Please address correspondence to:
Thambi Dorai, Ph.D
Departments of Urology, Medicine, Biochemistry & Molecular Biology
New York Medical College, Valhalla, NY 10595
E-Mail: thambi_dorai@nymc.edu

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ABSTRACT

In the course of studies aimed at the role of oxidative stress in the development of metastatic potential in the LNCaP-C4-2B prostate cancer progression model system, we found a relative decrease in the level of expression of the cytoplasmic nicotinamide riboside : quinone oxidoreductase (NQO2) and an increase in the oxidative stress in C4-2B cells compared to that in LNCaP or its derivatives C4 and C4-2. It was also found that C4-2B cells specifically shed large extracellular vesicles (LEV) suggesting that these LEVs and their cargo could participate in the establishment of the osseous metastases. The level of expression of caveolin-1 increased as the system progresses from LNCaP to C4-2B. Since NQO2 RNA levels were not changed in LNCaP, C4, C4-2 and C4-2B, we tested an altered cellular distribution hypothesis of NQO2 being compartmentalized in the membrane fractions of C4-2B cells which are rich in lipid rafts and caveolae. This was confirmed when the detergent resistant membrane fractions were probed on immunoblots. Moreover, when the LEVs were analyzed for membrane associated caveolin-1 as possible cargo, we noticed that the enzyme NQO2 was also a component of the cargo along with caveolin-1 as seen in double immunofluorescence studies. Molecular modeling studies showed that a caveolin-1 accessible site is present in NQO2. Specific interaction between NQO2 and caveolin-1 was confirmed using deletion constructs of caveolin-1 fused with glutathione S-Transferase (GST). Interestingly, whole cell lysate and mitochondrial preparations of LNCaP, C4, C4-2 and C4-2B showed an increasing expression of glutaminase (GLS, kidney type). The extrusion of LEVs appears to be a specific property of the bone metastatic C4-2B cells and this process could be inhibited by a GLS specific inhibitor BPTES, suggesting the critical role of a functioning
glutamine metabolism. Our results indicate that a high level of expression of caveolin-1 in C4-2B cells contributes to an interaction between caveolin-1 and NQO2 and to their packaging as cargo in the shed LEVs. These results suggest an important role of membrane associated oxidoreductases in the establishment of osseous metastases in prostate cancer.

INTRODUCTION

Prostate adenocarcinoma is recognized as the second leading cause of death in men in North America (1). Although the mechanisms by which prostate cancer is driven are not yet completely understood, aspects such as age, race, family history of prostate cancer, lifestyle, and diet are factors are under investigation (2-4). Prostate cancer incidence is tested on the basis of the prostate-specific antigen (PSA) blood test and histological stages (5). Early stages of prostate cancer exhibit cells that are only able to survive in an androgen-rich environment and hence they are often treated with androgen-deprivation therapies. While it is common that bony metastases are seen at first clinical diagnosis of prostate cancer in many men, there is also an increasing suspicion that the selective and stringent pressure imposed by the androgen ablation therapy on the residual prostate cancer cells might actually accelerate the development of more aggressive, metastatic and rapidly growing prostate cancers (6). This is particularly in part due to fact that the prostate cancer cells are adept at maintaining functional androgen receptor signaling even in an androgen-independent environment, to drive cancer growth and to acquire a metastatic advantage (6,7). Studies have been done to dissect the mechanisms that drive prostate cancer transition from androgen dependence to androgen
independence, in animal models (8,9). However, the complete molecular mechanism of prostate cancer progression is yet to be elucidated, and therefore, even the best available treatment for advanced prostate cancers often result in debilitating osseous metastases, often with a fatal outcome (10,11).

The LNCaP prostate cancer progression model, developed by Leland Chung's laboratory, systematically recapitulates the way in which prostate cancer develops and eventually metastasizes preferentially to the skeleton (12,13). Thus, this model system lends itself to systematic pre-clinical studies to understand the stages involved in prostate cancer progression to androgen independence and the establishment of bony metastases. Characterization of the LNCaP progression model revealed an increasing “bone cell-like” differentiation from LNCaP human prostate carcinoma cells to its derivatives C4, C4-2, and C4-2B. Interestingly, LNCaP and C4 are androgen dependent whereas C4-2 and C4-2B cells are androgen independent. This *in vitro* model mirrors the progression of human prostate cancer *in vivo* (12,13). In addition to being androgen independent, C4-2B cells have the propensity to metastasize to the bone. Bone is a restrictive and protective environment that is normally hostile to the growth and survival of cancer cells metastasizing from another organ. Therefore, in order for prostate cancer cells to metastasize in the bone, they mimic the properties of osteoblasts and osteoclasts, types which bone cells are made of, in order to survive, a phenomenon known as "osteomimicry". The classic "seed and soil" theory states that the soil, the bone microenvironment, determines the selectivity of seed, in this case the prostate cancer metastatic cell line C4-2B (14,15). Therefore, in order for the seed to take root, the
prostate cancer cells closely mimic the fertile soil of the local bony microenvironment by exhibiting osteomimicry.

Several epidemiological studies indicate that while the rate of occurrence of clinically indolent prostate cancer is similar worldwide, the rate of occurrence of clinically significant and metastatic prostate cancer is markedly lower in Asia than in Western populations (16). It has been proposed that these differences could be due, a large part, to the influencing factors such as diet and other nutritional factors rather than genetic variations because Asian men who adopt a Western life style show an increasing incidence of clinically significant disease (17). Apart from Asian men, incidence of prostate cancer among men is also significantly low in France which is due, a significant part, to a large intake of red wine, a natural source of resveratrol, leading to the concept of “French Paradox” (18,19). Work from our laboratories have focused for many years on the molecular mechanisms of androgen independence and bone metastasis in prostate cancer, with particular reference to the use of curcumin (used in Asian cuisine) and resveratrol (found in red wine) as significant anti-inflammatory, anti-oxidative agents and as nutritional modulators of cancer progression (20-24). Our earlier work on the mechanisms of androgen independence in prostate cancer cells has led to a better understanding of the function of the enzyme NRH: Quinone oxidoreductase (NQO2) in prostate carcinogenesis (25, 26). In this communication, the role of the enzyme NQO2 is investigated further with respect to its role not only in regulating oxidative stress but also in regulating prostate cancer metastasis and how glutamine metabolism influences all these processes. Our results highly suggest a heretofore uninvestigated role for the enzyme NQO2 in the genesis and function of large extracellular vesicles (LEV)s,
particularly in the modulation of the bony microenvironment in advanced prostate cancer through its specific interaction with caveolin-1 which is implicated in the genesis of hormone refractory and metastatic prostate cancer.

MATERIALS AND METHODS

Cell Culture:

The human prostate cancer cell lines used in this study are LNCaP, C4, C4-2 and C4-2B. Of these, the LNCaP cell line (as its fast growing version, FGC) was originally purchased from American Type Culture Collection (ATCC). The sublines C4, C4-2 and C4-2B derived from the parental LNCaP were originally developed and extensively characterized by Chung and his laboratory (12,13). These cell lines were procured from ViroMed Inc and preserved in liquid nitrogen and cultured as described previously (21, 22). The non-cancerous immortalized human prostate epithelial cell line RWPE-1 was purchased from ATCC and cultured in a medium containing keratinocyte serum free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor (Invitrogen, Inc).

Antibodies and reagents:

Antibodies to NQO2 (monoclonal) was purchased from Origene Technologies, Inc. Polyclonal antibody to Glutaminase (GLS) was from Novus Biologicals Inc. Antibodies to NQO1 (monoclonal) and β-actin (polyclonal) and caveolin-1 (polyclonal) were from Santa Cruz Biotechnologies, Inc. The cell permeable GLS inhibitor BPTES was purchased from Sigma-Aldrich, Inc.
**Analysis of oxidative stress inherent in un-induced prostate cancer cells:**

This was done using the CellROX green oxidative stress assay kit for live cells, purchased from Thermo Fisher Scientific, *Inc*. The four prostate cancer cell lines were plated in chambers in a 4-well chamber-slide format (Lab Tek II chamber slide system, Nalge- Nunc International). When the cells were around 70% confluent, the cells were treated with CellROX green reagent at a final concentration of 5µM and incubated at 37°C for 30 minutes. They were then washed thrice with 1X phosphate buffered saline (PBS), the chambers were removed and the slides were finally mounted using Vecta Shield antifade mounting medium supplemented with DAPI (Vector Labs, *Inc*) and cover slips. The slides were then visualized through a Nikon Eclipse 90i fluorescence microscope and images were captured at the excitation/emission wavelengths of 485 and 520nm respectively.

**Isolation and Reverse Transcription of RNA:**

Isolation of total RNA from all the 4 prostate cancer cells were done through the TriZol method, following manufacturer's protocols (Bio Rad, *Inc*) and quantitation was done by following its absorbance at 260 nm. The first strand synthesis was done using the oligo-dT primer and MuMLV-reverse transcriptase (New England BioLabs, *Inc*) using the standard protocol supplied by the manufacturer.

**Semi-quantitative PCR amplification of NQO1, NQO2 and β-actin transcripts:**

The design of the sense and anti-sense primers for the genes NQO1, NQO2 and β-actin (for expression control) was done exactly as described previously by Strassburg *et al* (27). NQO1, NQO2 and β-actin genes were amplified separately using the specific
primers, under the PCR conditions described by these authors, using the thermostable TaqMan DNA polymerase (Applied Biosystems, Inc) and Applied Biosystems Gene Amp PCR system 2400. One tenth of the total PCR product for each was run on a 1% agarose gel containing 1 μg/ml ethidium bromide and the gel was imaged through the gel documentation system. Under these conditions, NQO2, NQO1 and β-actin yielded a PCR product of 623, 366 and 202 bps respectively.

**Cell proliferation assays:**

RPMI-1640 culture medium supplemented with 2mM or 0.1 mM glutamine were added to 12 well plates with a final volume of 1ml per well and seeded with LNCaP, C4, C4-2 and C4-2B cells at day 0 at a density of 1X10^5 cells per well. Following cell attachment, growth medium with the same concentration of glutamine was replaced the next day and subsequently every 2 days. At day 6, the cells were trypsinized, suspended in 1ml of medium and the cells were counted using a hemacytometer to determine the total number of cells per well. All cell proliferation assays were done in duplicate.

**Immunoblot analysis:**

Whole cell lysates were prepared in lysis buffer consisting of 50 mM Tris-HCl buffer pH 8.0 supplemented with 1% Triton X-100, 150mM NaCl, 2mM sodium orthovanadate, 2mM PMSF, 50mM sodium β-glycerophosphate and HALT protease inhibitor cocktail at 1X concentration. Protein concentrations were determined by using the BCA assay (Thermo Scientific, Inc). Pre-determined quantities of lysate proteins were denatured with the complete reducing SDS-sample buffer (Sigma –Aldrich Inc) by boiling for 5 minutes. The denatured lysates were then resolved on Bio-Rad Criterion gels at either 8% or at
12% acrylamide concentrations. The resolved proteins were transferred to PVDF membranes (Bio-Rad). The membranes were blocked with 5% blotting grade defatted dry milk (Bio-Rad) in Tris-buffer-saline supplemented with 0.1% Tween-20 for 1 hr at room temperature. The blocked membranes were probed overnight at 4°C in the primary antibody solution at concentrations recommended by the manufacturer in TBS-Tween supplemented with 1% dry milk. After washing three times in TBS-Tween, the blots were probed with the corresponding secondary antibody (at 1:3000 dilution) conjugated with horse radish peroxidase for 1 hr at 4°C. After the final washes, the signal was developed with the enhanced chemiluminescence reagent Luminol (Santa Cruz Biotechnology, Inc) as recommended by the manufacturer and the bands were captured on to HyBlot ES autoradiography film (Denville Scientific, Inc).

**Molecular modeling for NQO1 and NQO2 and identification of potential caveolin-1 binding sites:**

This is done following the Pymol molecular modeling program, following specific instructions from the software for the target proteins NQO2 and NQO1.

**Isolation of detergent resistant membranes from prostate cancer cells:**

LNCaP, C4, C4-2 and C4-2B cells were cultured as described earlier in 60 mm plates and grown to 80% confluency. The cells were fractionated into detergent soluble and detergent resistant fractions as originally described by Hamaguchi and Hanafusa (28). Briefly, the CSK buffer used consists of 10 mM Pipes pH 6.8, 100 mM KCl, 2.5 mM MgCl$_2$, 1mM CaCl$_2$, 0.3 M sucrose, 1mM phenylmethylsulfonyl fluoride (PMSF), 1% Trasylol (Aprotinin), 1mM sodium orthovanadate, 10µM sodium molybdate, 1% Triton X-
Grown cells were washed with Tris-buffered saline and incubated with 0.5 ml of CSK buffer for 3 min on ice with gentle shaking every 30 sec. The supernatant was collected and the insoluble matrix structure that remained on the dish was again incubated for 1 min on ice with an additional 0.5 ml of fresh CSK buffer. The supernatants from the two incubations were pooled and used as the detergent soluble fraction. The residual structure remaining on the dish was collected with 0.3 ml of 1X RIPA buffer (Santa Cruz Biotechnologies, Inc) supplemented with 2mM sodium ortho-vanadate and 1X protease inhibitor cocktail (Santa Cruz) and used as the detergent resistant fraction. Both fractions were centrifuged at 15,000g for 30 min and the supernatants were used for analysis.

**Double immunofluorescence for probing caveolin-1 and NQO2:**

The four prostate cancer cell lines were cultures on Lab Tek II 4-well chamber slides as mentioned before. When they are 70% confluent, the slides with the chambers still intact were washed with serum free medium (RPMI-1640) three times and then incubated with 1 ml of the same serum free medium overnight at 37°C. The chambers were then washed with 1X PBS and fixed with an acetone:methanol mix (1:1 vol/vol) at -20°C for 20 min, the fixed cells were immersed in IX PBS and were processed for double immunofluorescence as follows. The chambers were treated with 5% donkey serum in 1X PBS supplemented with 0.5% Triton-X100 (blocking buffer which also permeabilizes the cells) for 1 hr at room temperature. The chambers were treated with the 1st primary antibody (polyclonal anti-caveolin-1, Santa Cruz Biotechnologies, Inc) in blocking buffer, at a dilution of 1:150), in a final volume of 150 microliters overnight at 4°C. After this treatment, the chambers were washed three times with 1X PBS, for 10 min each at room
temperature. The chambers were treated with the 1st secondary antibody, ie., Alexa-488 conjugated donkey anti-rabbit IgG (Invitrogen, Inc), diluted in 1X PBS (1:300) for 2 hrs at room temperature. The chambers were washed in IX PBS as described before and treated again with blocking buffer for 1hr at room temperature. The chambers were then treated with the 2nd primary antibody (monoclonal antibody to NQO2, Origene Technologies) in blocking buffer at a dilution of 1:150, overnight at 4°C. The next day, the chambers were washed with IX PBS and treated with the 2nd secondary antibody (Alexa 594-conjugated donkey anti-mouse IgG from Invitrogen, diluted in 1XPBS at a dilution of 1:300). For this secondary antibody treatment, the above diluted solution was centrifuged at 12000g for 30 min at 4°C to remove some fluorochrome crystals that interfere with the immunofluorescence analysis. The supernatant was used to treat the chambers for 2 hrs at room temperature. After this final treatment, the chambers were washed three times with 1X PBS to remove the excess fluorochromes. The chambers were now removed and the slide was mounted with VectaShield antifade (with DAPI) mounting medium (Vector Labs Inc) and sealed with a cover slip. The immunofluorescence of the two fluorochromes were captured using the Nikon fluorescence microscope as described earlier.

**GST-caveolin-1 (wt and deletion mutants) pull down assay:**

The full length caveolin-1 (cav-1, amino acids 1-178) and its deletion mutants (amino acids1-101 and amino acids 82-101) were fused with Glutathione S-Transferase (GST). Along with a GST only negative control, all the GST-constructs were a kind gift from the laboratories of D. Volonte and F. Galbiati of the University of Pittsburgh School of Medicine. All these constructs were transformed into _E Coli_ BL-21 strain and selected
for ampicillin resistance. Logarithmically growing cells were induced for recombinant protein expression through the addition of 5mM isopropyl thiogalactopyranoside (IPTG, Sigma-Aldrich). All the GST-cav-1 constructs were affinity purified by passing through glutathione (GSH)-agarose beads using the detergent sarcosyl for initial solubilization. Purified GST alone (negative control), GST-cav-1 (full length 1-178), GST-cav-1 mutant (1-101), GST-cav-1 mutant (82-101) proteins were immobilized onto GSH agarose beads and washed three times with TNET buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM EDTA and 1% Triton-X100 containing 1X Halt™ protease (Pierce Biotechnology, Inc) inhibitors. Whole cell extracts [prepared using radioimmunoprecipitation assay buffer, (RIPA) from Santa Cruz, Inc] from logarithmically growing LNCaP cells were treated with each of these GSH-agarose immobilized proteins in a batch format overnight at 4°C in TNET buffer. The unbound proteins were removed by brief centrifugation and the beads were washed three times with the same TNET buffer. The bound proteins were eluted and denatured with 2X SDS-PAGE denaturing buffer and subjected to gel electrophoresis under denaturing conditions. The resolved proteins were transferred to PVDF membranes as described above and probed with anti-NQO2 antibody (monoclonal, Origene Technologies Inc).

**Isolation of large extracellular vesicles (LEV)s:**

Large extracellular vesicles were purified by differential centrifugation from the conditioned medium prepared from the prostate cancer cell lines as well as the immortalized non-tumorigenic primary epithelial prostate cell line RWPE-1. For each cell line, two confluent 150 mm dishes were used (nearly 30 million cells). The cells were washed three times with sterile phosphate buffered saline (1X PBS) and incubated further
in serum free RPMI-1640 medium for 12 more hours. The conditioned medium (CM) from these serum starved conditions was removed from the cells and centrifuged at 300g for 10 minutes to pellet intact cells, which were discarded. The supernatant from this centrifugation was centrifuged at 1000g for 10 minutes to pellet cell debris. The supernatant from this centrifugation was filtered through a 0.22μm steri-flip filter unit (Millipore) with a PVDF membrane. The large extracellular vesicles retained by the filter was washed once with 1X PBS and re-suspended in 0.5 ml of the same buffer. Alternatively, the supernatant obtained after the 1000g centrifugation was processed again for ultracentrifugation in a SW 50.1 rotor in a Beckman Ultracentrifuge at 100,000g for 60 minutes. This ultracentrifugation step yielded a pellet which consisted of both LEVs and smaller sized EVs. For treatment of cells with a specific Glutaminase (GLS) inhibitor (BPTES), the respective cells grown in 60 mm dishes in rich medium to 70% confluency was treated with BPTES at a final concentration of 10 μM for 24 hours. At the end of this period, the cells were washed in 1X PBS and cultured in serum free medium in the presence of 10μM BPTES for a further period of 12 hours. Subsequent harvesting of LEVs from the BPTES treated conditioned medium was done through membrane filtration as described above.

**RESULTS:**

* Differential expression of NQO2 in the prostate cancer progression model system:

The relative levels of expression of the enzyme NQO2 and caveolin-1 were followed using the whole cell extracts of LNCaP, C4, C4-2 and C4-2B cells. As negative
and loading controls, the expression levels of the enzyme NQO1 as well as β-actin were also followed on the same gels by re-probing the blots. The results are shown in Figure 1A. It is evident that while caveolin-1 expression progressively increased, the level of NQO2 changed in a biphasic manner, first increasing from LNCaP to C4, peaking at C4-2 followed by a precipitous decrease at the C4-2B stage. These results, at the minimum, suggest that NQO2 is being unexpectedly reduced compared to caveolin-1 in C4-2B cells.

To obtain additional information on the discordant changes in NQO2 and caveolin-1 with respect to prostate cancer progression and metastasis, total RNA was prepared from these cells and mRNA expression corresponding to NQO1, NQO2 and β-actin was assayed by RT-PCR. In contrast to the immunoblots data shown in Figure 1A, relatively constant levels of mRNA expressions of NQO2 as well as NQO1 were observed (Figure 1B). These PCR results suggest that the relatively lower level of expression of NQO2 in the bone metastatic C4-2B cell line is not likely mediated by a transcriptional mechanism but could be attributed to a post-translational event that effectively lowers the intracellular concentration of this enzyme.

**The C4-2B metastatic progression is accompanied by an increase in levels of oxidative stress:**

Since NQO2 participates in the detoxification of reactive oxygen species including intracellular semiquinones, its decreased expression during LNCaP progression to C4-2B could result in cell stage-dependent change in intracellular oxidative stress. This possibility was tested using the CellROX Green oxidative stress assay kit (Invitrogen, Inc). The assay involved a cell permeable fluorogenic probe which displays a weakly fluorescent signal in the reduced state and exhibits a bright green photo-stable
fluorescence upon oxidation by intracellular reactive oxygen species (ROS) allowing it to bind to DNA with an absorption/emission maximum of 485/520 nm. Thus, the fluorescence intensity of this probe is an indirect measurement of the intracellular ROS levels. Results in Figure 1C show a progressive increase in the ROS levels from LNCaP to C4-2B similar to what has been demonstrated in several cancer cell systems (29,30).

**A sub fraction of NQO2 is associated with detergent resistant membranes of prostate cancer cells:**

Although the reduced expression of NQO2 could account for the increased intracellular ROS during LNCaP progression to the metastatic stage, the mechanisms that might underlie the lowered level of NQO2 remain to be determined. We hypothesized that NQO2 while largely cytoplasmic acting as a ROS detoxification enzyme may acquire a novel structural and functional role by becoming compartmentalized in membranes. To investigate this hypothesis, we prepared detergent soluble and detergent resistant fractions of LNCaP, C4, C4-2 and C4-2B cells using established protocols and probed for NQO2 expression by immunoblot analysis. Interestingly, we found a significant localization of NQO2 in the detergent insoluble membrane fraction and that the relative proportion of membrane associated NQO2 increased as cells progressed from LNCaP to C4-2B (Figure 2A). For loading controls, equivalent amount of estimated protein was loaded for lanes 1 and 2 in this Figure. For the detergent resistant membrane fraction which is devoid of most protein, one-tenth of the extracted membrane fraction was loaded in this study (lane 3). Since C4-2B cells show the most phenotypic changes such as increased oxidative stress, increased caveolin-1 expression and decreased NQO2 expression, we wished to characterize the detergent resistant membrane fractions from
this cell line further, the results of which are shown in Figure 2B. As expected, the β-actin levels are equivalent in the whole cell lysate (lane 1) and the detergent soluble (lane 2) fractions and there was a significant reduction in its expression in the detergent resistant (lane 3) membrane fraction. Other membrane markers such as Flotillin-1 and caveolin-1 showed higher expressions in the detergent resistant membrane fraction. Interestingly, the cytoplasmic marker oncogene p60c-src did not localize with the detergent resistant membrane fraction which served as a negative control (28). These results suggest that there is a compartmentalization of NQO2 in detergent resistant, caveolae rich membrane fractions of C4-2B cells.

**Molecular modeling reveals potential caveolin-1 binding sites in NQO1 and NQO2:**

Since the detergent resistant fractions isolated are known to be rich in lipid rafts and caveolae, we further hypothesized that NQO2 may be associated with the caveolae and specifically with caveolin-1. To determine whether caveolin-1 binding sites are intrinsically present in the NQO2 protein, we performed molecular modeling of NQO2. For comparison, the sites potentially available on a related quinone oxidoreductase NQO1 was also analyzed as a negative control. Analysis of the NQO2 and NQO1 protein sequences using the PyMol modeling software revealed that there are two caveolin-1 consensus binding sites in NQO2 and one such binding site in NQO1. Interestingly, we found that all caveolin-1 binding domains (CBD) are directed towards the scaffolding domain of caveolin-1 (CSD) which mediates direct protein-protein interactions between caveolin-1 and a variety of signaling molecules. These signaling molecules possess the CBD consensus sequence ΦXΦXΦXΦXΦ, ΦXΦXΦXΦXΦ, where Φ represents an aromatic amino acid and X represents any amino acid (31, 32). Thus,
NQO2 has two putative CBD between amino acids 149 and 157 and between 175 and 182 while the sequence of NQO1 between amino acids 100 and 107 of NQO1 also conforms to the consensus CBD. However, the sequences in NQO2 (149-157) and in NQO1 (100-107) are buried in the core of the protein, near the isoalloxazine ring of the flavin binding site and hence, not likely to be available for binding to caveolin-1. By contrast, the sequence in NQO2 (175-182) is located at the periphery and spatially available for potential protein-protein interactions with caveolin-1 (Figure 2C).

**Glutathione-S-Transferase-fused caveolin-1 construct pull-down assays confirm that NQO2 interacts with caveolin-1:**

To investigate whether caveolin-1 is a binding partner for NQO2, we performed pull-down assays using a series of caveolin-1 deletion constructs fused with glutathione-S-transferase (GST) and non-denaturing whole cell lysates from LNCaP prostate cancer cells. Figure 3 shows that NQO2 is a *bona fide* caveolin-1 binding protein and that at the minimum, the scaffolding domain of caveolin-1 (amino acids 82-101, fused to GST) was sufficient for binding to NQO2. Many other investigators have found that this scaffolding domain of caveolin-1 also interacts with several significant signal modifiers such as β-catenin, thioredoxin reductase-1, Nrf2 *etc*, to keep them in an inhibited state, thus regulating signal flow.

**Both caveolin-1 and NQO2 are specific components of the cargo carried by the large extracellular vesicles (LEVs) of bone metastatic C4-2B cells:**

Currently, there is a large body of evidence showing that cancer cells interact and communicate with normal and healthy cells such as fibroblasts present in the tumor...
microenvironment (TME). Cancer cells accomplish this mainly by their ability to release extracellular vesicles that appear to play the role of molecular trojan horses in that they carry important signaling proteins and other cargo to be transferred to neighboring cells in order to transform them. While the release of smaller exosomes (<100 nm) appear to be common to both normal cells and cancer cells, the release of large extracellular vesicles (LEV) or large “oncosomes” (>10 µM in diameter) appear to be specific to cancer cells (33-35). In particular, prostate cancer cells are known to typically release LEVs which allow them to carry critically significant signaling proteins and other cargo such as microRNAs etc across tissue barriers (36). Recent work of Di Vizio et al showed that these LEV release from prostate cancer cells into the blood appear to correlate with advanced metastatic disease and caveolin-1 was identified as the serum LEV biomarker (37). Therefore, these observations prompted us to explore the possibility of the release of these LEVs in the well characterized prostate cancer progression model system of LNCaP and C4-2B. Since it is already known that caveolin-1 is an integral component of these LEVs, we hypothesized that NQO2 could also be a cargo carried by these LEVs to modulate the oxidative stress of the neighboring cells. Our studies indicate that the release of LEVs is greatly increased in the C4-2B cells as opposed to the parental LNCaP cells and that the extrusion of both caveolin-1 and NQO2 could be visualized in the LEVs as shown by double immunofluorescence studies (Figure 4A). As a negative control, we also looked for the disposition of the related oxidoreductase NQO1 and found that it was not released in the LEVs from C4-2B cells (Figure 4B).

The C4-2B cells are glutamine addicted as compared to LNCaP, C4 and C4-2:
We performed cell proliferation studies in rich as well as glutamine deficient medium after seeding a constant number of cells in 6-well plates. Compared to LNCaP, C4 and C4-2 cells, C4-2B showed a 60% reduction in proliferation when grown in glutamine deficient medium. Similar results were obtained when C4-2B cells were grown in rich medium in the presence of 10 µM BPTES, the specific inhibitor of GLS (Figure not shown). Our results suggest that these C4-2B cells are critically dependent on the presence of glutamine for their metabolism, survival and proliferation, compared to its parental derivatives.

The formation and extrusion of LEVs from C4-2B cells are critically dependent upon glutamine metabolism, particularly Glutaminase (GLS activity):

Interestingly, we also found that the release of these LEVs is critically dependent upon the presence of glutamine and its metabolism, particularly the pathway mediated by the kidney type Glutaminase (GLS). Figure 5A shows a significant reduction in LEV production from the highly bone metastatic C4-2B cells when the enzyme GLS was specifically inhibited by the compound BPTES. Further supporting our observation, studies from other laboratories indicate that proteins enriched and exported in LEVs included enzymes involved in glucose and glutamine metabolism. Recent results of Minciacchi et al show that the glutamine metabolism was altered in cancer cells exposed to LEVs. These authors showed in their DU-145 metastatic prostate cancer system, the GLS enzyme was highly and uniquely expressed in the LEV cargo fraction (38). This prompted us to investigate whether GLS enzyme was also present in the LEVs identified in the C4-2B bone metastatic system. Figure 5B shows that the GLS extrusion in the LEVs budding from C4-2B cells (which is not seen in the parental LNCaP, C4 and C4-2
cells, Figure not shown) as visualized by immunofluorescence. This GLS extrusion in the LEVs from C4-2B cells could be completely inhibited by BPTES as seen in Figure 5B.

**Parallel immunoblot analyses confirm the immunofluorescence pattern of C4-2B cells:**

To gain further supporting evidence for the presence of NQO2, caveolin-1 and GLS in these LEVs, we also performed immunoblot analyses on the isolated LEVs from different stages of prostate cancer, as exemplified by the LNCaP progression model system. Figure 6A shows the results of immunoblots when the whole cell lysates (WCE) and the large extracellular vesicles (LEV) fractions were run on the same blot and probed with the respective antibodies such as NQO1, RhoC, β-actin and Glutaminase (GLS). Of these, the first two proteins served as negative controls. NQO1 is only seen in the WCE fraction and not in the LEV fraction, a pattern also seen in the immunofluorescence studies (Figure 4B). The RhoC protein is exclusively cytoplasmic and hence not seen in the LEV fraction. A small pool of β-actin protein could be seen in the LEV fraction which is an indication that the LEV blebbing and release in the C4-2B system is critically dependent on the cytoskeletal organization just below the plasma membrane and that a fraction of the actin filament proteins such as β-actin is also extruded in these LEVs. This phenomenon is not seen when parallel studies were done on exosomes as opposed to LEVs (data not shown). β-actin shedding in LEVs could also be seen in the breast cancer system as reported independently by another study (39). Thus, β-actin shedding in several cancer systems appears to be specific to LEVs. Coincidentally, the extrusion of β-actin could be inhibited to a large extent by the GLS specific inhibitor BPTES. We also confirmed the presence of the enzyme GLS in these LEVs from C4-2B cells, the extrusion
of which is again dependent upon glutamine metabolism, particularly GLS activity, as evidenced by the inhibition of its own shedding by the GLS specific inhibitor BPTES. These observations give a new meaning for the self-serving nature of glutamine addiction in cancers. In Figure 6B, we further characterize the extrusion of vesicle specific proteins such as Flotillin-1 and the cancer specific proteins under investigation in this study, such as NQO2 and caveolin-1. Flotillin-1 expression, as a marker for the LEV fraction, could be seen in the LEVs of C4-2B cells in the absence of BPTES and this expression is greatly reduced in the presence of BPTES. The presence of the proteins that are the focus of this study, namely NQO2 and caveolin-1 could be seen in the LEV fraction of C4-2B cells in the absence of BPTES while their expression is greatly reduced in the presence of BPTES at the concentration used, a pattern that was consistently seen in the double immunofluorescence studies.

**The osteomimicry of the C4-2B prostate cancer cells is dependent upon glutamine metabolism:**

Finally, since we had characterized the link between glutamine metabolism and the extrusion of LEVs in the highly bone metastatic C4-2B prostate cancer cell line, we wished to find out whether the well established osteomimicry properties of these cells could also be influenced by the inhibition of GLS by BPTES. Figure 6C shows the immunoblot analysis of the osteomimetic marker proteins that are known to be expressed by the C4-2B cells. Specifically, we followed the expression of bone sialoprotein (BSP, an osteoblastic marker), receptor activator for NF-kB (RANK, an osteoclastic marker) and parathyroid hormone related protein (PTHrP, another osteoclastic marker) against the background expression of the β-actin proteins in the C4-2B whole cell extracts in the
absence (-) and in the presence (+) of 10μM BPTES. This Figure shows a remarkable reduction in the level of expression of these osteomimetic marker proteins when these cells are cultured in the presence of BPTES. These results reveal that the glutamine metabolism (as shown through GLS inhibition by BPTES) is critically required for both LEV production as well as the exhibition of osteomimetic properties by these prostate cancer cells. Since the establishment of osseous metastases by these cancer cells are critically dependent upon glutamine metabolism, we propose that a specific inhibition of GLS mediated metabolism and the glutamine addiction of these C4-2B cells could be therapeutic and could lead to an inhibition of bone metastases.

**DISCUSSION:**

This study reports the first observation that the enzyme NQO2, largely regarded so far as a Phase II detoxification enzyme present in the cytoplasm of the cells, has a membrane component with its membrane associated function. Its localization in the caveolae and its specific interaction with caveolin-1 suggests that it is specifically regulated by caveolin-1, similar to the way caveolin-1 suppresses the oncogenic signaling molecules such as src, Akt, Nrf2, thioredoxin-1, β-catenin etc (40-42). The concept of duality in caveolin-1 function, both as a tumor suppressor as well as a tumor promoter in a context dependent way, is well known (43-45). But, our observation that caveolin-1 and NQO2 form part of the cargo in the large extracellular vesicles suggests that once caveolin-1/NQO2 is out of their native caveolar compartment, it may lose its potential tumor suppressor activities and acquire tumor promoting (gain of function) activities when one considers the dual nature of these proteins (43-45). Specifically, our observations
highly suggest that the extra-caveolar localization of caveolin-1 and NQO2 may in fact have a gain of function, possibly in modifying the tumor microenvironment and in preparing the pre-metastatic niche for the establishment of the metastatic lesion (according to the seed and soil hypothesis) particularly enhancing the propensity to establish bone metastases by the C4-2B prostate cancer cells. These capacities are just the opposite of their tumor suppressive (in the case of caveolin-1) and the ROS and quinone detoxifying (in the case of NQO2) functions (46, 47). However, these hypotheses need to be further investigated. Since NQO2 is known to have a dual role as a reactive oxygen species (ROS) detoxifier as well as a ROS generator, it is possible that this enzyme may function, in a context dependent way, as a tumor promoter, similar to caveolin-1 (48,49). Therefore, any strategy to inhibit the formation of these LEVs in addition to the naturally available anti-NQO2 strategies would be clinically therapeutic and it would inhibit the formation of bone metastases in advanced prostate cancer patients. Our studies highlight the obligatory requirement of enhanced glutamine metabolism in the display of several osteomimetic properties of C4-2B prostate cancer cells as well as the production of LEVs by these cells. It is possible now to envision how we can interfere with the establishment of organ specific metastases. Since the cancer cells from advanced prostate cancer patients as well as defined cell lines such as C4-2B display enhanced growth factor receptor expression and activation; activate their pro-inflammatory NF-kB pathways, express both osteoblast as well as osteoclast specific proteins, express prostate specific antigen, endothelin-1, Cbfa1 transcription factors etc, these cells have more in common with the bone microenvironment when compared with that of the liver or lung (12,13, 21, 22 ). Thus, exhibition of osteomimetic properties and
the reliance on enhanced glutamine metabolism for the exhibition of these properties are significant and amenable to therapeutic targeting in a combinatorial manner. One aspect that is not addressed in our studies is the enhanced synthesis of lipids that is necessary to form the membranes of LEVs extruded from C4-2B prostate cancer cells. Since these advanced prostate cancer cells (as well as breast cancer cells and other cancer systems) show a propensity to increase their lipid synthesis, giving rise to the established concept of the “lipogenic” phenotype, our studies on the release of LEVs from C4-2B cells correlate with such enhanced lipid synthesis requirement. Thus, inclusion of a specific fatty acid synthase (FASN) inhibitor as an adjuvant could be therapeutically beneficial (50,51).

Our studies suggest that metastasis and glutamine metabolism could be closely related and regulated in prostate cancer cells and more specifically, in the bone metastatic prostate cancer cells to achieve their invasive goals. Several other published studies directly or indirectly support this relationship (52-57). Therefore, anti-GLS therapy with the use of specific GLS inhibitors (which are currently under clinical trials) should be considered as therapies to inhibit the formation of bone metastases in advanced prostate cancer patients as it is likely to break the vicious cycle of bone destruction and bone formation (21,22,58). At the least, they should be considered as adjuvant therapies along with the mainstay therapies for late stage prostate cancer patients exhibiting osseous metastases such as the use of nitrogen containing bisphosphonates (NBPs).

Furthermore, our studies emphasize the importance of the interaction between caveolin-1 and NQO2. NQO2 joins other bona fide interactors for caveolin-1 such as Nrf2, Akt, c-src, eNOS, Thioredoxin reductase etc. One common denominator among all
these interactions is that caveolin-1 keeps all these signaling proteins under check by suppressing their activities which is facilitated by cavin-1. Studies by several investigators indicate that caveolin-1 expression was induced without the presence of cavin-1 in advanced prostate carcinoma and in this context enhanced caveolin-1 expression activated Akt signaling in prostate cancer, emphasizing again the duality of the function of caveolin-1 (59-61). Our future studies will investigate the role played by cavin-1 in the extrusion of these LEVs. NQO2 fits well into the paradigm of LEV cargo containing non-caveolar caveolin-1 influencing the stromal compartment. Yet, more studies are needed to test this hypothesis. Studies from other laboratories highly suggest that these LEVs bypass the extracellular and tissue barriers to fuse with the target non-cancerous cell types (in the stromal compartment) and influence their metabolic and signaling pathways (62, 63).

Our studies are also important from a translational point of view. For example, there are several reports that suggest a role of a sudden oxidative burst in otherwise silent micrometastases that allow them to “wake up” from tumor dormancy. Based on the studies presented here and considering the fact that NQO2 can produce reactive oxygen species (ROS) under special circumstances, it is tempting to speculate that such events may be the “trigger” or a wake-up mechanism for prostate cancer micrometastases, a mechanism which could well be used by other cancers such as breast cancer (64, 65). Other than its potential role in tumor dormancy, these results also suggest that the expression and cellular partitioning of NQO2 and caveolin-1 in LEVs could participate in the membrane lipid peroxidation induced oxidative stress and membrane reorganization. A correlation may therefore exist between membrane associated oxidoreductases,
increased oxidative stress and metastatic potential. Thus, the dual nature of NQO2 function, located mainly in the cytoplasm while at the same time a significant fraction of which is associated with caveolin-1 at the membrane may underscore the important role of this enzyme in the establishment of bone metastases.

The interaction between caveolin-1 and NQO2 might also be useful in the research on melatonin. It is well known that melatonin is an established inhibitor of cancer metastasis, although the relevant mechanisms are not yet clear. Since NQO2 has a melatonin binding site, it would be very interesting to find out how melatonin binding influences caveolin-1 binding and vice versa and the associated consequences (66, 67). These studies may have relevance in the inhibition of cancer metastasis in general.

While there is an established relationship between metabolism and metastasis, the role of cancer cell metabolism in the establishment of organ specific metastasis such as bone is more specialized than what we have envisioned. For example, if the cancer cells (bone metastatic prostate cancer cells) exhibit osteotrophic factors such as Cbfa1/RUNX2, PTHrP1, osteocalcin, osteoprotegerin (OPG) etc, their reprogrammed glutamine metabolism will facilitate the establishment of osseous metastases. If the cancer cells express certain hepatotrophic factors, their glutamine addicted metabolism will help in the establishment of liver metastases. The same concept might be true for other cancer systems such as breast cancer. Therefore, reprogramming the glutamine metabolism of these cancer cells might be the least common denominator among the establishment of all these metastases. Hence, breaking this fundamental glutamine addiction may well impede the establishment of organ specific metastases for several cancer systems. Since reprogrammed glutamine metabolism has been shown to be
important for the formation of large extracellular vesicles (LEVs, as more lipid synthesis is needed) and since these LEVs are known to interact with the tumor microenvironment (TME) and possibly facilitate the formation of the pre-metastatic niche etc, it is reasonable to hypothesize that inhibition of glutamine addiction may well prevent the formation of organ specific metastases. Figure 7 summarizes all the information that is gleaned from this study, integrating the conventional (as a Phase II detoxifying enzyme) as well as the non-conventional role of NQO2 (as a stabilizer of the tumor suppressive protein p53) (68, 69), its possible role on the membrane, its release in LEVs and the possibility of these NQO2/caveolin-1 containing LEVs in the modulation of tumor microenvironment.

Our results lend further support for the previously published results on the role of caveolin-1 expression in prostatic hyperplasia, prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma. Overexpression of caveolin-1 and extracellular caveolin-1 have been linked to androgen independence in prostate cancer. In fact, suppression of caveolin-1 expression induces androgen sensitivity in hormone refractory and metastatic prostate cancer cells in a mouse model system. In further support, caveolin-1 is also known to regulate hormonal independence through lipid synthesis and abnormal signaling of the androgen receptor (70-73). The secretion of LEVs and their cargo content are topics of intense investigation in several cancer systems. Our finding that the oxidoreductase enzyme NQO2 piggybacking on caveolin-1 and released extracellularly in LEVs adds a potentially therapeutic significance for the overall role for oxidoreductases in the modulation of the tumor microenvironment (TME) and in the establishment and maintenance of bone metastases in prostate cancer. It is also possible that these LEVs (and their cargo capable of modulating redox systems as shown in our studies) can further
contribute to the modulation of metabolism in adjacent cancer cells and stromal cells in close vicinity (74). Quite possibly, a similar phenomenon may function in other solid tumor systems also. Further studies are needed to explore these interesting possibilities. Our results give a molecular explanation for the significance of overexpression of caveolin-1 and its externalization with regards to how it may influence hormone refractoriness and bone metastasis in a metastatic progression model system. This behavior is greatly aided by an enhanced dependence on glutamine metabolism, which facilitates enhanced lipid synthesis (needed for the formation of LEVs) through glutaminase (GLS), glutamate dehydrogenase (GDH), reductive carboxylation of α-ketoglutarate (α-KG) through isocitrate dehydrogenase (IDH), citrate lyase and finally fatty acid synthase (FASN) as shown in Figure 7. This paradigm integrates the role of enhanced glutamine metabolism, enhanced lipid synthesis and lipid metabolism in the establishment of organ specific metastases (75-78).

Finally, our results suggest a glutamine/GLS mediated metabolic reprogramming in the LNCaP model system as it progresses toward C4-2B is an integral component of the force driving the metastatic process. Based on the results of our studies, a combinatorial strategy using anti-metastatic therapies such as nitrogen containing bisphosphonates (NBPs) and anti-Glutaminase therapy, or alternatively, a cocktail consisting of naturally occurring plant compound based anti-NQO2, anti-GLS and anti-FASN drugs could be considered for treating advanced prostate cancer patients with bone metastatic complications. We would like to suggest that the release of caveolin-1, GLS and NQO2 etc in these LEVs could make them an ideal diagnostic and prognostic marker (in the serum) for advanced prostate cancer patients. LEVs in these cancer
systems are known to pack a multitude of other oncogenic proteins, regulatory microRNAs etc. Thus, the extrusion of a specific protein such as GLS or NQO2 may be indicative of the severity of the disease.

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FIGURE LEGENDS:

Figure 1A:

Relative levels of expression of NQO2, NQO1, caveolin-1 and β-actin in the LNCaP prostate cancer progression model system. Characteristically, the level of expression of NQO2 is decreased in C4-2B cells when the whole cell extracts were analyzed. Whereas, the expression levels of caveolin-1 showed a progressive increase in this model system. This apparent inverse relationship between these two proteins formed the basis of our further investigations. As a negative control, the expression levels of a related oxidoreductase NQO1 showed no changes in this progression model system.

Figure 1B:

RNA expression pattern for NQO2, NQO1 and β-actin in the LNCaP prostate cancer progression model system. The specific primers designed generated a band of 623 bp (
base pairs) for NQO2; 366bp for NQO1 and 202 bp for β-actin. See Materials and Methods for the design of the primers and experimental details.

**Figure 1C:**

Oxidative stress analysis in the LNCaP prostate cancer progression model system, using the CellROX oxidative stress analysis kit. This figure shows significantly increased levels of oxidative stress in C4-2B cells as opposed to the parental LNCaP cells.

**Figure 2A:**

A subpopulation of NQO2 is associated with the detergent resistant membrane fraction of prostate cancer cells. Immunoblot probed with NQO2 antibody. 1: Total lysate; 2: Detergent soluble membrane fraction; 3: Detergent resistant membrane fraction.

**Figure 2B:**

Further characterization of the whole cell lysate, detergent soluble and detergent resistant fractions from the bone metastatic C4-2B cell line with respect to membrane markers Flotillin-1, caveolin-1. As negative controls, the markers such as β-actin and p60c-src were also included. Lane 1: whole cell lysate; Lane 2: detergent soluble membrane fraction; Lane 3: detergent resistant membrane fraction. See text for further details.

**Figure 2C:**

The potential caveolin-1 binding domains in NQO2 and NQO1 as revealed by PyMol modeling analysis. The caveolin-1 binding domains are indicated in red and blue. The externally located caveolin-1 binding domain in NQO2 is depicted in blue. See text for details.
Figure 3:

GST-pulldown assay: The recombinant proteins from deletion constructs of Caveolin-1 fused to Glutathione-S-Transferase (GST) were prepared from the respective bacterial clones and used to pull down the interacting proteins from the lysates of LNCaP prostate cancer cells. The eluted proteins were then run on a denaturing protein gel and probed with the NQO2 antibody as described earlier. The use of the recombinant deletion mutant proteins of caveolin-1 allows for the study of the minimum sequence/structural requirement of caveolin-1 (ie., the sequence between amino acids 82 and 101, also called the caveolin scaffolding domain, CSD) that is required to interact with a partner, ie., NQO2.

Figure 4A:

Release of large extracellular vesicles (LEVs) from C4-2B cells. LNCaP, C4, C4-2 and C4-2B cells were grown on 4 well chamber slides. Semi-confluent cells were then fixed in methanol:acetic acid mixture and processed for double immunofluorescence after blocking the slides with 5% normal donkey serum in 1XPBS and 0.5% TritonX-100. A Caveolin-1 rabbit polyclonal antibody (Santa Cruz Biotechnologies, Inc) and a monoclonal antibody against NQO2 (Origene Technologies, Inc) were used, each at 1:150 dilution. Invitrogen’s AlexaFluor-488 conjugated to donkey anti-rabbit IgG was used to bind to the caveolin-1 primary antibody and AlexaFluor-594 conjugated to donkey anti-mouse antibody was used to bind the NQO2 primary antibody. Processing and washing and final mounting were done according to manufacturer’s protocol (Vector Labs, Inc). The final mounting medium (Vector Labs, Inc) also contained the dye DAPI, specific
for nuclear staining. The large extracellular vesicles that are shedding out of C4-2B cancer cells can be seen (white arrows).

Figure 4B:

As a negative control to the immunofluorescence studies done for Figure 4A, single immunofluorescence studies were done on the same cells grown in chamber slides using a monoclonal antibody for the oxidoreductase NQO1, using a secondary antibody conjugated to AlexaFluor-594, as shown for Figure 4A.

Figure 5A:

Glutaminase dependency of LEV production from C4-2B cells. Cells were grown in chamber slides in the absence (A) and in the presence of the cell permeable glutaminase (GLS) inhibitor BPTES at a final concentration of 10 µM. The shed LEVs in this Figure are denoted by white arrows.

Figure 5B:

Presence of the Glutaminase enzyme (GLS) in the LEVs arising from C4-2B, in the absence of BPTES (A) and in the presence of 10µM BPTES (B). A polyclonal antibody for GLS was used in these immunofluorescence studies along with the AlexaFluor 488 conjugated secondary antibody as described earlier. The GLS in LEVs can be seen (white arrows).

Figure 6A:

Immunoblot analysis of biomarker proteins that are packaged in the LEVs that arise from the RWPE-1, LNCaP, C4, C4-2 and C4-2B cells. WCE: Whole cell lysate; LEV: large
extracellular vesicles. In (A), expression levels of the oxidoreductase NQO1, the cytoplasmic RhoC, β-actin and Glutaminase (GLS) were followed in the absence (-) and in the presence of 10 μM BPTES.

**Figure 6B:**

The expression levels of the LEV marker Flotillin-1, the enzyme NQO2 and Caveolin-1 were followed both in the absence (-) and in the presence (+) of 10 μM BPTES.

**Figure 6C:**

C4-2B cells show decreased osteomimetic properties when grown in medium supplemented with the glutaminase (GLS) inhibitor BPTES (10μM). In this immunoblot the expression levels bone sialoprotein (BSP, an osteoblastic marker), RANK (receptor activator for NF-kB, an osteoclastic marker), parathyroid hormone related protein (PTHrP, an osteoclastic marker) were followed against a backdrop of the expression levels of β-actin as loading control.

**Figure 7:**

A diagrammatic sketch of the impact of reprogrammed glutamine metabolism in regulating the formation of bone metastases in prostate adenocarcinoma. The duality of the function of NQO2 as a generator of oxidative stress as well as a stabilizer of p53 (in a non-enzymatic fashion) is also depicted. The balance between these two processes, along with the extent to which the tumor is addicted to glutamine and the level of fatty acid synthase (FASN) activity may ultimately determine the cancer cell’s propensity to establish organ specific metastasis. Several other strategies are available to modulate glutaminase activity, such as the use of organo-selenium compounds. All these factors
may promote the formation of an organ specific (in this case bone metastatic) niche. As described in the text, the balance of these processes may also help determine the extent of tumor dormancy. Further studies are needed to examine all the hypotheses proposed.

REFERENCES:


15. Langley RS, Fidler IJ. The seed and soil hypothesis revisited: The role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer* 2011; 128:2527-2535.


64. Gay LJ, Malanchi I. The sleeping ugly: Tumor microenvironment's act to make or break the spell of dormancy. *Biochimica Biophys Acta* 2017; 1868:231-238.


