



The bone metastatic niche enhances GJA1-20k expression and mitochondrial remodeling in metastatic prostate cancer cells

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ARTICLE INFO

Keywords:

Prostate cancer

GJA1-20k

Metastasis

Hypoxia

Bone microenvironment

Mitochondria

ABSTRACT

Bone metastases are a major cause of morbidity in advanced prostate cancer (PCa), where tumor cells adapt to a hypoxic and osteoblast-rich microenvironment. Here, we investigated how endosteal niche conditions regulate GJA1-20k expression and mitochondrial dynamics in metastatic PCa cells. We demonstrate that osteoblastic-conditioned medium (ObCM) combined with hypoxia preferentially increase GJA1-20k expression over full-length Cx43, without changes in GJA1 mRNA levels. This result correlates with a redistribution of mitochondria from perinuclear to peripheral regions. Interestingly, by co-immunoprecipitation experiments, we show that GJA1-20k interacts with mitochondria. Finally, stable overexpression of GJA1-20k in PCa cells was sufficient to reproduce this mitochondrial remodeling. These findings identify GJA1-20k as a stress-adaptive effector that links translational reprogramming to metabolic plasticity within the bone metastatic niche, highlighting new therapeutic opportunities to target PCa dormancy and progression.

1. Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men and ranks fifth among cancer-related causes of death globally [1]. PCa is notably characterized by a high occurrence of bone metastases associated with severe skeletal-related events (SRE) resulting from disruptions in bone formation and resorption. Unlike many other cancers, PCa particularly exhibits a risk of metastasis even after initial treatment due to tumor dormancy of disseminated tumor cells. During this process, disseminated tumor cells remain quiescent before potentially reactivating. Following radical prostatectomy, approximately 30–40% of patients with intermediate-risk prostate cancer may experience biochemical recurrence during long-term follow-up, consistent with the long-term persistence of residual tumor cells that may remain dormant for extended periods [2]. Within the bone marrow, disseminated PCa cells can colonize two distinct microenvironments, the

perivascular and the osteogenic niche [3]. The latter, composed mainly of osteoblastic lineage bone cells and matrix components, acts as the main site for secondary metastases [4]. Soluble factors secreted by osteoblasts, together with osteoclasts, endothelial cells and immune cells, influence tumor dormancy and reactivation. This osteogenic niche is also characterized by low oxygen levels (~1% O₂), modulating gene expression, metabolic adaptation and stress response in PCa cells [5].

Bone metastasis formation is a complex, multifactorial process involving diverse molecular and cellular actors. Among them, connexin43 (Cx43), a structural protein of gap junctions, has been increasingly recognized for its involvement in PCa metastasis through both channel-dependent or independent functions. In PCa patients, Cx43 displays a specific expression signature in bone metastases with levels varying throughout PCa progression [6]. Its impact is dual, Cx43 may exert tumor-suppressive effects in initial stages of PCa and also promote migration and invasion, thereby facilitating metastatic potential in

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<https://doi.org/10.1016/j.bbrc.2026.154336>

Received 9 May 2026; Received in revised form 14 July 2026; Accepted 22 July 2026

Available online 23 July 2026

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advanced stages [6]. We previously showed that the bone microenvironment modulates Cx43 expression, enhancing membrane localization and promigratory function in LNCaP cells exposed to ObCM. However, in androgen-insensitive PC-3 cells derived from bone metastases, Cx43 trafficking to the plasma membrane is impaired [7], leading to cytoplasmic retention [8] and loss of responsiveness to bone-derived factors [6]. Immunohistochemical analysis of bone metastases further confirms this cytoplasmic accumulation of Cx43 in advanced PCa, suggesting that subcellular localization, not just expression level, governs Cx43 functional impact within the metastatic niche [6].

Recently, a short isoform of Cx43, arising from alternative translation and corresponding to the end part of the fourth transmembrane domain and the entire carboxyl-terminal (CT) domain of Cx43, has been identified and termed GJA1-20k [9]. This isoform acts as an actin-capping protein, stabilizing actin filaments and facilitating microtubule-directed trafficking of full-length Cx43 to the plasma membrane, essential for gap junction assembly [10,11]. Additionally, GJA1-20k localizes to the outer mitochondrial membrane, where it promotes a protective form of mitochondrial fission that maintains ATP production and reduces oxidative stress [12,13]. In the cardiac model, GJA1-20k is recognized as an endogenous stress response protein upregulated under ischemic or ischemia/reperfusion conditions, where it supports cardioprotection by maintaining intercellular coupling between myocytes and attenuating cardiac hypertrophy [10,12,14]. Similarly, its overexpression in astrocytes promotes neuronal viability and recovery after traumatic brain injury in rat [15]. In the cancer field, although GJA1-20k expression has been observed in several cell lines such as PCa cells, its functional role remains largely unexplored [16,17]. James et al. (2018) [18] demonstrated in a mouse mammary gland epithelial cell line (NmuMG cells) that GJA1-20k expression decreases relative to full-length Cx43 during TGF β 1-induced Epithelial-to-Mesenchymal Transition (EMT). This transition is accompanied by increased trafficking of full-length Cx43 to the plasma membrane as previously observed in cardiac cells. In PCa cells, we previously showed that GJA1-20k is endogenously expressed in LNCaP cells and upregulated under ObCM treatment [6] or rapamycin exposure, a specific inhibitor of mTOR [19].

Since GJA1-20k is known to act as a stress-response protein that modulates mitochondrial dynamics in cardiac and neural tissues, and considering the hypoxic and metabolically challenging environment of the osteogenic bone niche, the present study sought to determine how hypoxia and ObCM affect GJA1-20k expression in the bone metastatic PC-3 cell model. Building on recent findings showing that GJA1-20k plays a role in mitochondrial fission and subcellular redistribution, mechanisms crucial for metastatic plasticity and dormancy, this study investigates how these niche conditions and GJA1-20k overexpression impact mitochondrial distribution in metastatic PCa cells. We demonstrate that GJA1-20k is upregulated by ObCM and hypoxia, interacts with mitochondria and is sufficient to drive their peripheral redistribution in metastatic PCa cells, highlighting its role in stress-adaptive remodeling. Our results provide new insights into how the bone microenvironment regulates PCa cell adaptation through GJA1-20k, potentially informing novel therapeutic strategies targeting metastatic progression and dormancy.

2. Material and methods

2.1. Cell cultures

The human prostate cancer PC-3 cell line was purchased from the American Type Culture Collection (ATCC RRID:CVCL_0035) and authenticated by short tandem repeat profiling (Microsynth). Stable overexpression of GJA1-20k in PC-3 cells was achieved using a plasmid previously generated by Smyth and Shaw (2013) [9], and obtained from Addgene. Briefly, PC-3 cells were transfected with pcDNA3.2-GJA1-20K-V5 plasmid (Plasmid #49859 - referred to as PC3-GJA1-20k) using FuGene HD transfection reagent following the manufacturer's instructions (Promega). Corresponding control cell line was

generated by transfecting PC-3 cells with the respective empty vector and will be referred to as PC3 mock. 48 h after transfection, cells were subjected to selection with 1 mg/mL G418 (Geneticin; Sigma-Aldrich) for two weeks. Stably transfected clones were maintained under continuous selection in medium with 1 mg/mL G418. All clones of PC-3 cells were cultured at 37°C in a humidified 5% CO₂ incubator in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, ThermoFisher Scientific) supplemented with 200 mM GlutaMAX™, 0.1 g/L sodium pyruvate, 4.5 g/L D-glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin.

Primary murine osteoblastic cells (OB) were isolated from calvaria of 3-5 days-old mice by sequential digestions with trypsin and collagenase, as described previously [8]. OB cells were expanded to confluency at 37°C in phenol red-free DMEM supplemented with 20% FBS, and then seeded and maintained for 5 days in differentiating medium (phenol red-free DMEM, 10% FBS and 10 mM sodium β -glycerophosphate (Sigma)). After 5 days, the osteoblastic-conditioned medium (ObCM) was collected, centrifuged sequentially at 300 $\times$ g and then at 14,000 $\times$ g for 10 min and stored at -20°C until use.

When indicated, PC-3 cells were cultured in hypoxic condition (1% O₂) using a hypoxylab chamber (Oxford Optronix) and/or in the presence of ObCM.

2.2. Western blot analysis

Western blotting was performed as previously described by Boucher et al. (2020) [6]. Briefly, total protein extracts were obtained by lysing cells in ice-cold RIPA buffer supplemented with proteases inhibitors. Cell lysates were centrifuged at 12,500 $\times$ g for 10 min at 4°C to remove debris and protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories). Equal amounts of proteins were mixed with 5X Laemmli sample buffer, resolved on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Membranes were blocked for 1 h at room temperature (RT) in TBS-T (Tris-buffered saline with 0.1% Tween-20) containing 5% (w/v) non-fat dry milk, and then incubated overnight at 4°C with primary antibodies (see Table 1) diluted in blocking buffer. After washing with TBS-T, membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse or anti-rabbit, 1:10,000). Immunodetection was performed using Luminata Forte chemiluminescent substrate (Merck) and signals were captured using the LAS-3000 imaging system (Fujifilm). Relative band intensities were quantified by densitometric analysis using ImageJ software (version 1.52).

2.3. Immunocytofluorescence

PC-3 cells were cultured at low density on sterile glass coverslips for different times and then fixed in ice-cold methanol/acetone (1:1) for 10 min. After blocking with PBS containing 1% bovine serum albumin (BSA) and 1% Triton X-100 at RT for 1 h, PC-3 cells were incubated overnight at 4°C with specific primary antibodies (see Table 1) diluted 1:250 in blocking buffer. Negative controls were performed by omitting the primary antibody. After washing with PBS, PC-3 cells were incubated with Alexa Fluor 488 anti-mouse secondary antibodies (1:1000 in blocking buffer) for 2 h at RT. Coverslips were mounted with vectashield-DAPI fluorescent mounting medium (Vector Laboratories) to counterstain nuclei. Fluorescence imaging was performed using an Olympus FV3000 confocal microscope. 1024x1024 pixels images were acquired with 1.5 NA: $\times$ 100 objective lens. 3D optical sectioning of 0.3 µm were driven with step Z-axis motor.

2.4. Analyze of mitochondrial distribution

We developed a Fiji macro to analyze Z-stack projections of TOM20 immunostaining images. The macro performs maximum intensity projection, segments nuclei using automatic thresholding, and allows

Table 1

List of antibodies used in the study.

Target	Source	Dilution	Application	Reference	Supplier
Connexin43 C-terminal	Mouse	1/1000, 5% BSA	Western blotting	#610062	BD Transduction Laboratories
GAPDH	Mouse	1/10,000, 5% milk	Western blotting	#5G4	Hytest
TOM20	Mouse Ig2a	1/250	Immunostaining	sc-17764	Santa cruz
		1 µg/500 µL cell lysate	Co-IP		
DAPI	---	1/1000	Nuclear staining	32670	Sigma
HIF1-alpha	Mouse	1/1000	Western blotting	ab2185	Abcam
GAM-HRP	Goat	1/5000, 5% milk	Western blotting	A0168	Sigma
GAM-488, Alexa Fluor	Goat	1/250	Immunostaining	A11029	Invitrogen

manual definition of line ROIs for intensity profile analysis. This streamlined workflow facilitates quantitative assessment of mitochondrial markers in Z stacks. The macro script is available in the [Supplementary Fig. S2](#).

2.5. Co-immunoprecipitation (CoIP)

Co-immunoprecipitation (CoIP) experiments were performed using 1 mg of total protein extracted as described in section 3.2. Cell lysates were diluted in 500 µL of CoIP buffer (50 mM Tris HCl pH7.4, 5 mM MgCl₂, 150 mM NaCl and 0.05% (v/v) NP-40) and samples were incubated overnight at 4°C with the appropriate primary antibody and 50 µL of glycerol (10%). The following day, to precipitate the immunocomplexes, an incubation with 30 µL of protein A-Sepharose beads was conducted for 2 h at 4°C with gentle agitation. Immunocomplexes were collected by centrifugation and eluted by boiling in Laemmli buffer. The resulting samples were analyzed using Western blotting. Appropriate controls, including immunoprecipitation with isotype control antibodies or beads alone, were performed and have confirmed the specificity.

2.6. Real-time quantitative PCR

Total RNA extraction, reverse transcription and RT-qPCR were performed as previously detailed [20]. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene and relative expression levels were calculated using the comparative $\Delta\Delta C_t$ method. Specific primers were synthesized by Eurogentec: Cx43-forward, 5'-GCT-GGT-GGT-GTC-CTT-GGT-GTC-3'; Cx43-reverse, 5'-CAG-GAG-GAG-ACA-TAG-GCG-AGA-GG-3'; GAPDH-forward, 5'-TGC-ACC-ACC-AAC-TGC-TTA-GC-3'; GAPDH-reverse, 5'-GGC-ATG-GAC-TGT-GGT-CAT-GAG-3'.

2.7. Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using tetramethylrhodamine ethyl ester (TMRE) as previously described by Antigny et al. (2009) [21]. Briefly, cells were incubated for 5 min at RT with 10 nM TMRE. Fluorescently labeled cells were then imaged using a confocal microscope to monitor mitochondrial membrane potential.

2.8. ROS production

Intracellular reactive oxygen species (ROS) production was quantified using the fluorescent probe carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-DCFDA; Sigma-Aldrich, #21884). Cells (2×10^3 per well) were seeded in black 96-well plates and cultured for 48 h. After incubation with 10 µM carboxy-DCFDA for 1 h at 37°C, cells were washed, lysed in RIPA buffer, and fluorescence was immediately measured (Ex/Em: 485/530 nm).

2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 8.0, GraphPad Software, USA). Data are presented as mean $\pm$ the standard deviation (SD) from at least three independent

experiments. Normality was assessed using the Shapiro-Wilk test. Unpaired *t*-tests were used to compare two experimental groups. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was applied. Statistical significance was accepted at *p*-values <0.05 (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

3. Results

3.1. GJA1-20k is selectively upregulated by osteoblast-conditioned medium and hypoxia

To investigate how specific conditions of the bone niche influence GJA1-20k expression in metastatic prostate cancer cells, we first examined the effect of osteoblast-conditioned medium (ObCM) on PC-3 cells. In the presence of ObCM, GJA1-20k expression progressively increased over time, with a marked upregulation by 5-fold from 24 to 96 h of culture (Fig. 1A and B). In contrast, cells maintained in control medium did not exhibit significant changes in GJA1-20k levels. Notably, full-length Cx43 (FL-Cx43) levels remained relatively stable throughout the time course, with only a slight significant increase at 48 h. These data indicate that ObCM selectively induces GJA1-20k expression in PC-3 cells, independently of FL-Cx43, suggesting a niche-specific regulatory mechanism.

To assess the impact of hypoxia, a defining feature of the osteogenic bone niche, on GJA1-20k expression, PC-3 cells were cultured under low oxygen conditions (1% O₂) for 48 and 72 h. Hypoxia induction was first validated after 4 h by increased HIF1-alpha expression (Fig. S1), and the same hypoxic condition was maintained for the 48 h experiment. After 48 h, hypoxia induced notable cell morphological changes, characterized by cell elongation consistent with a cellular stress response (Fig. 2A). Western blot analysis revealed a two-fold increase in GJA1-20k expression after 48h (Fig. 2B) which was not maintained after 72 or 96 h of hypoxia (Fig. 2C), indicating a transient induction. Reoxygenation for 24 h following hypoxic exposure reversed this effect (Fig. 2B), restoring GJA1-20k expression to baseline, while no upregulation was observed under normoxic conditions (Fig. 2B and C). Importantly, full-length Cx43 remained unchanged at the same time points suggesting a specific regulation of the GJA1-20k expression. Transcript analysis confirmed that GJA1 mRNA level was unaffected by hypoxia (Fig. 2D), supporting a post-transcriptional mechanism involved in the specific increase of GJA1-20k expression. Altogether, these results demonstrate that GJA1-20k is transiently upregulated in response to hypoxic stress in metastatic PCa cells.

To more closely replicate the conditions of the osteogenic bone niche, PC-3 cells were simultaneously exposed to ObCM and hypoxia. This dual stimulation induced a synergistic increase in GJA1-20k expression levels after 48 h (Fig. 3A and B). Quantitative analysis revealed a 5-fold increase under hypoxia alone and a 16-fold increase with ObCM, whereas the combined treatment resulted in a 32-fold elevation in GJA1-20k expression. In contrast, full-length Cx43 levels exhibited only a modest increase under ObCM conditions by 2.5-fold and 2-fold in normoxia and hypoxia respectively. Consequently, these data demonstrate that GJA1-20k becomes the predominant isoform

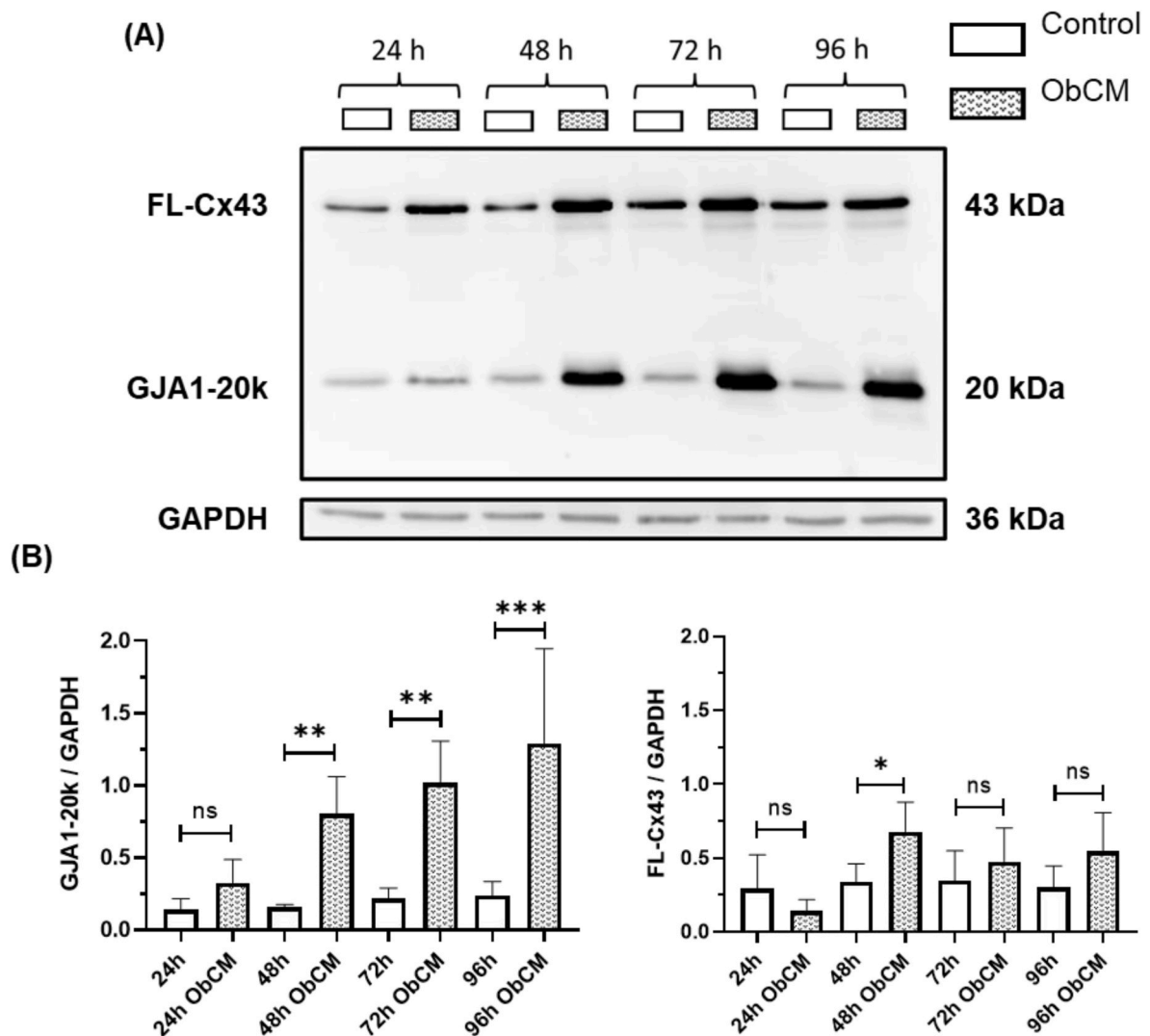


Fig. 1. GJA1-20k is upregulated in response to osteoblastic-conditioned medium in metastatic prostate cancer cells.

(A) Representative immunoblot and **(B)** densitometric quantification of GJA1-20k and full-length Cx43 (FL-Cx43) expressions in PC-3 cells cultured for 1 to 4 days in control medium (ctl) or osteoblastic-conditioned medium (ObCM) (N = 3, each performed in duplicate). GAPDH was used as loading control. *p < 0.05 to ***p < 0.001.

under simulated endosteal niche conditions (82%) compared to perivascular niche conditions (26%) (Fig. 3C).

3.2. GJA1-20k interacts with mitochondria and its overexpression leads to reorganization of the mitochondrial network

Metabolic adaptation is a critical determinant of cancer cells survival and metastatic progression. In cardiac and brain models, GJA1-20k has been shown to regulate mitochondrial number and subcellular distribution through its interaction with the outer mitochondrial membrane. Given the upregulation of GJA1-20k under hypoxia and ObCM, we hypothesized that this isoform may contribute to mitochondrial remodeling in metastatic PCa cells.

To test this hypothesis, we first investigated the mitochondrial distribution in PC-3 cells exposed to hypoxia and/or ObCM conditions using TOM20 immunostaining (Fig. 4). Under normoxic conditions and in control culture media, TOM20 signal was predominantly localized to the perinuclear region, consistent with baseline mitochondrial organization [22]. In contrast, stress conditions, hypoxia and ObCM treatment, induced a clear reduction in overall TOM20 fluorescence intensity (Fig. 4A), accompanied by a pronounced redistribution of mitochondria

toward the cell periphery (Fig. 4B). Quantitative analysis, independent of mitochondrial mass, confirmed a significant increase in peripheral mitochondrial localization relative to the perinuclear zone. Interestingly, co-immunoprecipitation assays revealed that GJA1-20k co-precipitated with TOM20 in PC-3 cells, confirming a physical interaction between the two proteins (Fig. 4C). Notably, this interaction was markedly enhanced following exposure to hypoxia and ObCM, conditions that also induced GJA1-20k upregulation and peripheral mitochondrial redistribution.

To determine whether GJA1-20k overexpression is sufficient to drive the mitochondrial redistribution, PC-3 cells were stably transfected with a GJA1-20k expression construct inducing a 3.4-fold increase in expression (Fig. 5A). Similarly to hypoxic and ObCM conditions, a clear lowering in overall TOM20 fluorescence intensity was observed (Fig. 5B). In control cells, TOM20 signal remained concentrated in the perinuclear region. In contrast, GJA1-20k-overexpressing cells exhibited a marked shift in mitochondrial localization toward the cell periphery, closely mimicking the pattern observed under hypoxic and ObCM conditions (Fig. 5C). Quantitative analysis confirmed a significant increase in peripheral mitochondrial distribution in GJA1-20k-transfected cells compared to controls. These findings demonstrate that GJA1-20k alone

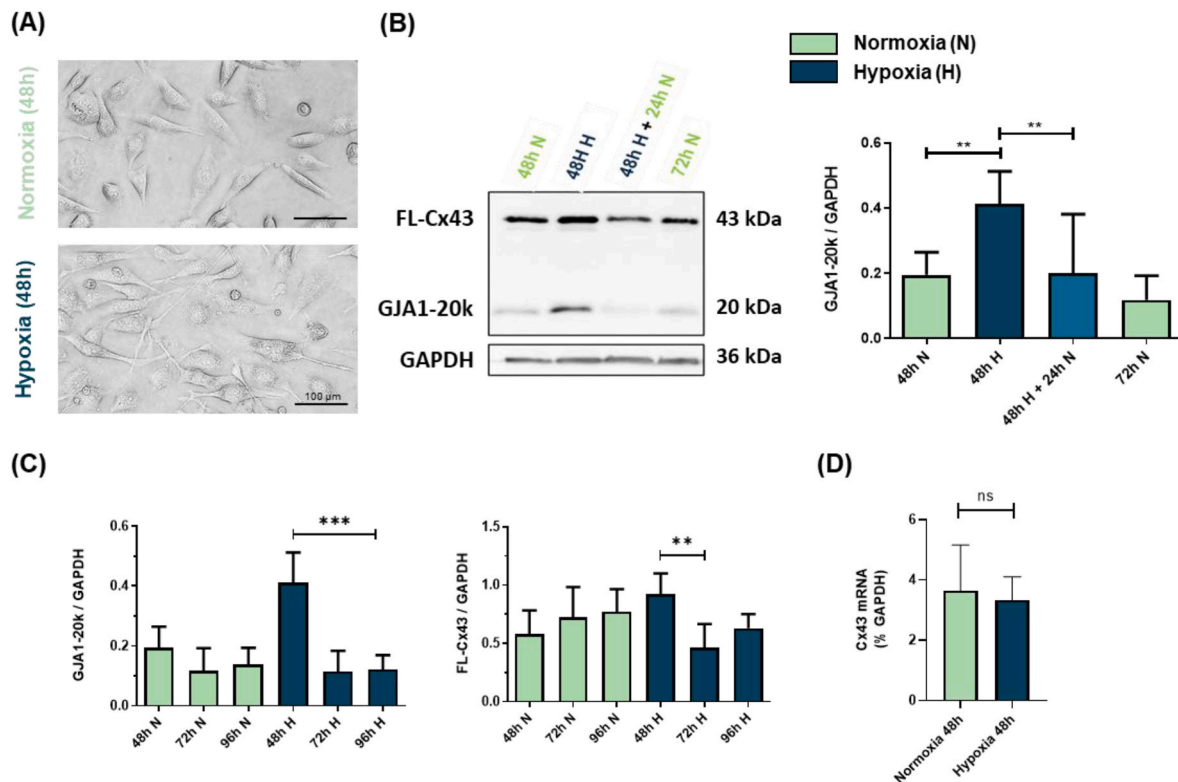


Fig. 2. GJA1-20k is upregulated in response to hypoxia in metastatic prostate cancer cells.

(A) Phase-contrast micrographs of PC-3 cells cultured for 2 days under normoxic or hypoxic conditions (1% O₂). Scale bar: 100 μ m. (B) Representative immunoblot and densitometric quantification of GJA1-20k expression in PC-3 cells after 2–3 days of culture under normoxia (N) or hypoxia (H) (N = 3, each performed in duplicate). (C) Relative quantification of full-length Cx43 (FL-Cx43) and GJA1-20k protein levels in PC-3 cells (N = 3) over time under normoxic or hypoxic conditions. (D) GJA1 mRNA expression level quantified by RT-qPCR in PC-3 cells after 48 h of culture under normoxia or hypoxia (N = 3). GAPDH was used as loading control or reference gene. **p < 0.01 to ***p < 0.001.

is sufficient to induce mitochondrial repositioning in metastatic PCa cells, supporting its role as a mediator of stress-adaptive remodeling within the bone microenvironment. To determine whether this GJA1-20k-mediated reorganization correlates with functional changes in mitochondrial bioenergetics and baseline redox states, we next evaluated mitochondrial membrane potential and intracellular reactive oxygen species (ROS) production in the stably transfected cells. Live-cell imaging using the TMRE fluorescent probe revealed a significant decrease in mitochondrial membrane potential in GJA1-20k-overexpressing PC3 cells compared to mock-transfected PC3 cells (Fig. 5D). Consistent with this shift toward attenuated mitochondrial activity, quantification of intracellular oxidative status using the carboxy-DCFDA probe demonstrated a concomitant and significant reduction in basal ROS production in GJA1-20k-overexpressing cells (Fig. 5E). Taken together, these data demonstrate that the peripheral network redistribution driven by GJA1-20k is accompanied by a distinct functional metabolic adaptation, characterized by reduced membrane potential and dampened oxidative stress.

4. Discussion

Most patients with late-stage prostate cancer display metastasis at distant sites, predominantly in the bone, which are correlated with shorter survival. The bone microenvironment offers a uniquely supportive niche for prostate cancer cells, providing both structural shelter and a rich supply of growth factors that promote survival, dormancy, and eventual reactivation [23]. However, to persist in this niche, PCa cells have to adapt microenvironmental stressors such as hypoxia and paracrine signaling from osteoblasts. Understanding the molecular mechanisms that govern this stress adaptation is critical to uncovering

how PCa cells survive and re-emerge. In this context, GJA1-20k, a truncated isoform of connexin43, appears to be a promising candidate since GJA1-20k is known to be upregulated during ischemia/reperfusion in the heart and during brain injury, leading us to investigate its role in PCa cells exposed to bone metastatic niche conditions.

To mimic the soluble factor profile of the bone niche, we used a murine model of osteoblastic-conditioned media (ObCM). While this *in vitro* system does not fully recapitulate the complex *in vivo* microenvironment, it has been extensively characterized both by our group and others [6]. We found that ObCM upregulates GJA1-20k expression in PC-3 cells, consistent with our previous findings in LNCaP cells [6], suggesting that osteoblast-derived factors potentially modulate the non-canonical translation in metastatic PCa cells. Osteoblasts are known to regulate gene expression and protein synthesis via paracrine signaling in PCa cells, promoting diverse phenotypes including proliferation, invasion or quiescence [24–26]. However, to our knowledge, there are currently no studies demonstrating that osteoblast-secreted factors or extracellular vesicles can directly induce internal ribosome entry sites (IRES)-mediated translation. Previous works have shown that inhibition of the PI3K/AKT/mTOR, Mnk1/2 or EGFR pathways increases endogenous GJA1-20k expression in both primary and cancer cells [9,27]. This raises the possibility that osteoblast-derived factors may suppress these signaling pathways in PCa cells, thereby promoting GJA1-20k expression. Notably, direct interaction between PCa cells and osteoblasts induces expression of TANK binding kinase 1 (TBK1), a non-canonical I κ B kinase (IKK) family member, which inhibits mTOR signaling, contributing to cellular dormancy [28]. Elucidating the molecular basis of GJA1-20k regulation in this context, specifically through direct quantification of IRES activity via reporter assays and the characterization of upstream candidates like the PI3K/AKT/mTOR or Mnk1/2 pathways,

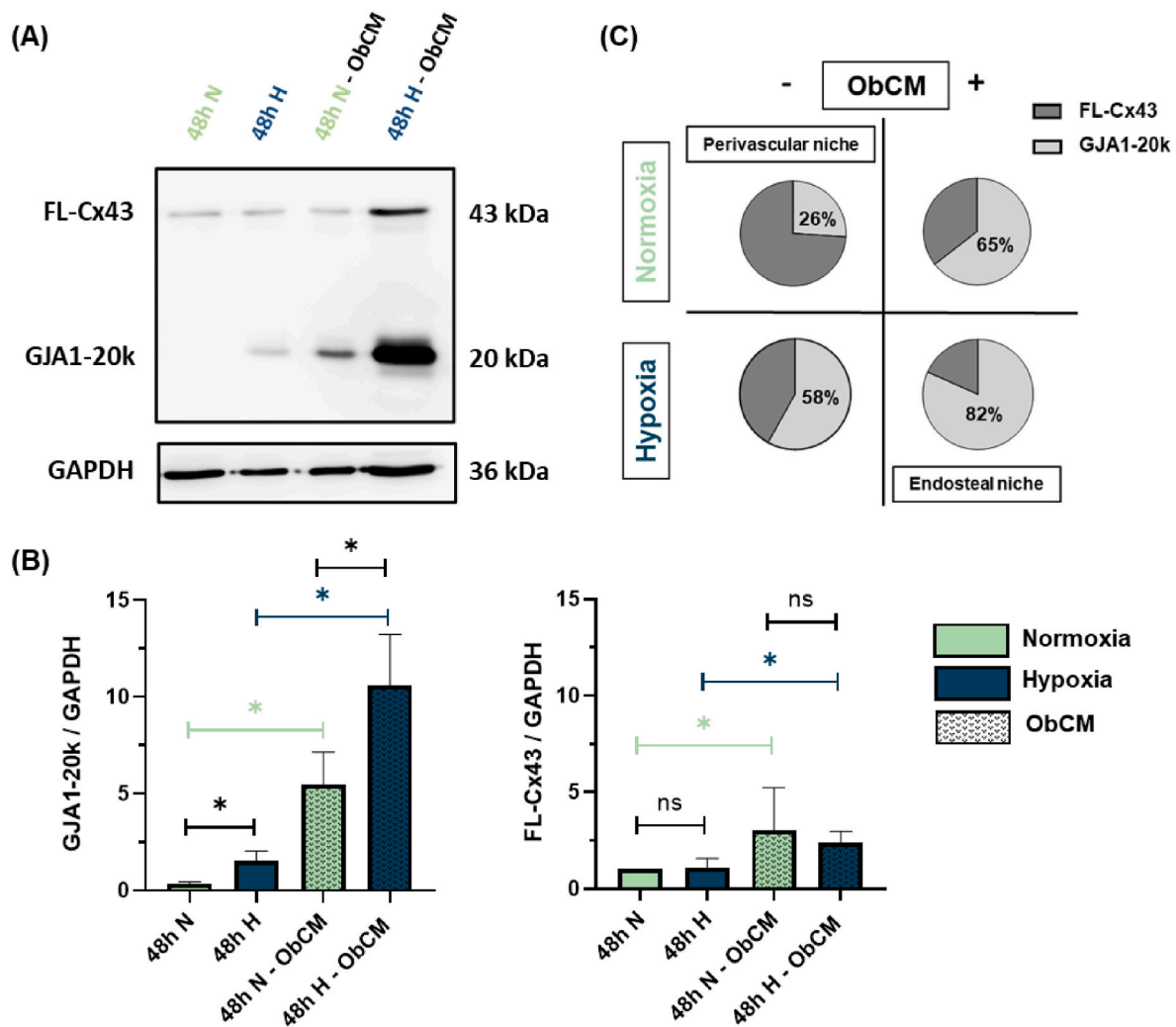


Fig. 3. GJA1-20k is highly upregulated in response to bone microenvironment in metastatic prostate cancer cells.

(A) Representative immunoblot and (B) densitometric quantification of GJA1-20k and full-length Cx43 (FL-Cx43) in PC-3 cells cultured for 2 days under normoxic (N) or hypoxic (H; 1% O₂) conditions, with or without exposure to osteoblastic-conditioned medium (ObCM) (N = 4, each performed in duplicate). GAPDH was used as loading control. (C) Relative distribution (%) of FL-Cx43 and GJA1-20k protein levels under Normoxia, Hypoxia with or without ObCM conditions (based on immunoblots in Fig. 3A). Percentages indicate the proportion of GJA1-20k. *p < 0.05.

will be essential to fully uncover how osteoblasts regulate non-canonical translation and drive metastatic adaptation within the osteogenic niche.

This regulation is likely not restricted to the interaction between bone cells and the metastatic PCa cells. The hypoxic conditions, a hallmark of the bone microenvironment, represents an additional stress signal that may act in concert with secreted factors to activate cap-independent translation pathways. Consistent with this idea, we observed that GJA1-20k is transiently induced under hypoxic conditions in metastatic PCa cells, independently of GJA1 transcript levels.

These findings suggest that hypoxia, like ObCM, promotes the translational upregulation of GJA1-20k, consistent with previous reports describing GJA1-20k upregulation during ischemic or hypoxic stress in non-malignant tissues [29]. Other studies have shown that hypoxia is a stress condition known to block cap-dependent translation through suppression of the eIF4F complex, while simultaneously promoting cap-independent initiation via IRES [28] in order to initiate the translation of specific mRNAs that encode proteins involved in angiogenesis (e.g. VEGF-A), metabolic adaptation (e.g. HIF-1 alpha) and survival (e.g. c-Myc) in order to protect the cells [30–32].

As a consequence of ObCM treatment, we observed a previously unreported redistribution of mitochondria toward the cell periphery into the PCa cells. This remodeling may be driven by cytoskeletal

reorganization, as osteoblast-secreted factors are known to activate Rho GTPases, as Rac1 [6], or actin dynamics in prostate cancer cells which are critical for metastasis [33,34].

In parallel, hypoxia is also known to trigger profound changes in mitochondrial dynamics in cancer cells, including fragmentation and redistribution, as part of a metabolic shift toward glycolysis and localized ATP supply [35,36]. Peripheral positioning of mitochondria has been linked to increased cell motility and invasion, as it supports focal adhesion turnover and actin remodeling at the leading edge [37,38]. Our observation that hypoxia induces peripheral mitochondrial redistribution in PC-3 cells aligns with the spatial dynamics described in these invasive phenotypes. While direct functional cell migration and invasion assays are required to definitively establish whether this spatial reorganization directly accelerates metastatic trafficking, the structural remodeling highlights a clear response to niche-specific factors. Beyond driving structural reorganization, hypoxia and bone-derived soluble factors are known to induce profound alterations in mitochondrial bioenergetics and cellular redox status. Confirming such a functional metabolic adaptation in our model, TMRE analysis and ROS production assays demonstrated that GJA1-20k upregulation significantly altered mitochondrial membrane potential and modulated intracellular redox homeostasis. This coupled functional remodeling likely represents an

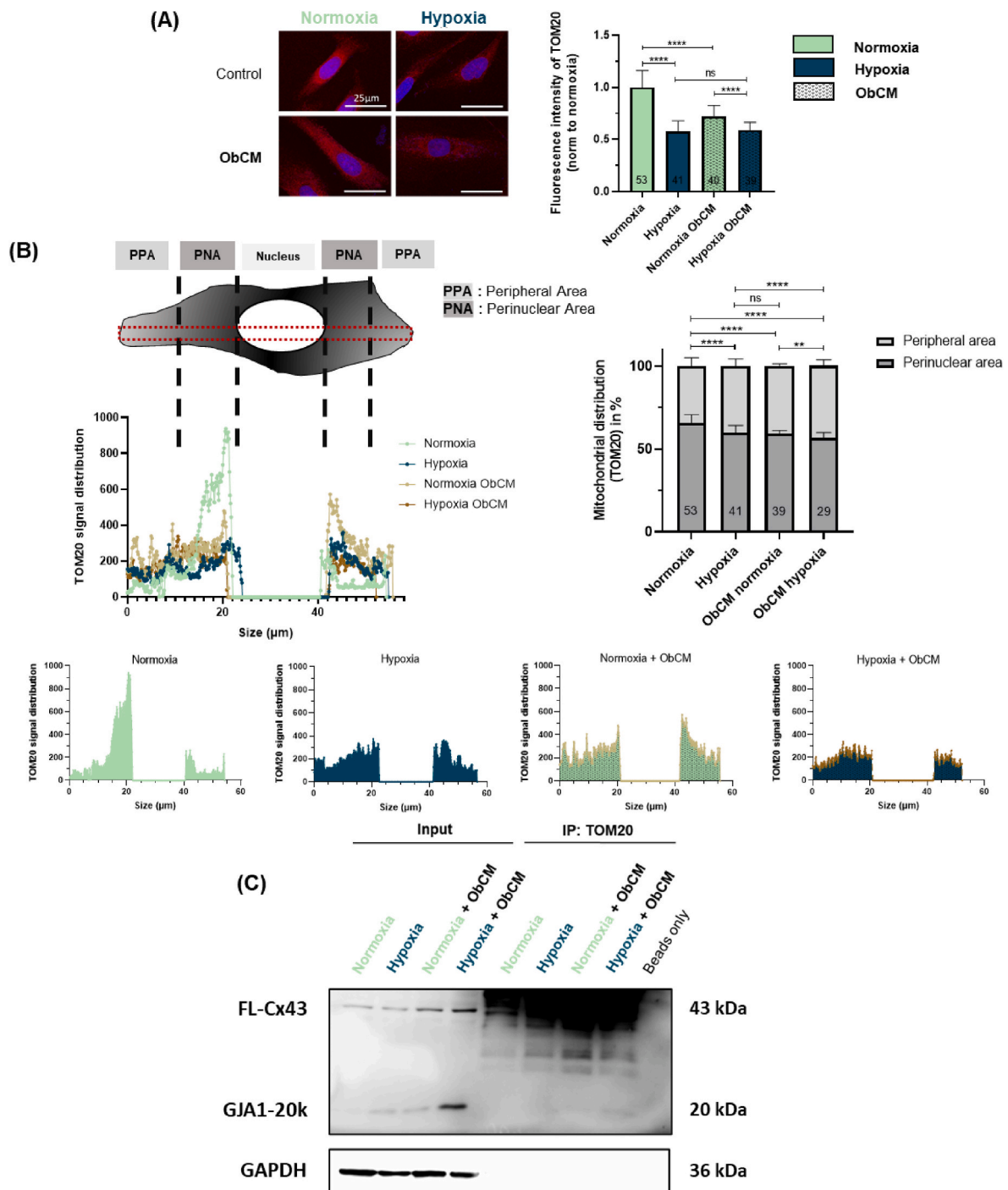


Fig. 4. Mitochondrial network rearrangement in metastatic prostate cancer cells cultured in bone stress conditions.

(A) Representative images of mitochondrial network and quantification of mitochondria in PC-3 cells (TOM20 immunostaining) cultured for 2 days under hypoxia and/or ObCM conditions (blue = nucleus, red = TOM20), Scale bar = 25 μm. **(B)** Quantification of mitochondrial distribution in peripheral versus perinuclear regions under normoxia or hypoxia with or without ObCM, based on TOM20 immunostaining. Numbers inside the bars indicate the total number of analyzed cells. **(C)** Coimmunoprecipitation (Co-IP) assays showing interactions between TOM20 and GJA1-20k or FL-Cx43 in PC-3 cells exposed to normoxia or hypoxia with or without ObCM (N = 5). GAPDH was used for input control. A beads-only control was included to assess non-specific binding and GAPDH was used for input control. **p < 0.01, ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adaptive mechanism providing microenvironmental stress tolerance, similar to the GJA1-20k-dependent protection observed in cardiac models during ischemia-reperfusion injury [12,39]. Future investigations utilizing extracellular flux analysis will further refine how these variations precisely rewire the bioenergetic profiles of metastatic prostate cancer cells within the bone niche.

The concomitant increase in GJA1-20k/TOM20 interaction under hypoxia combined with ObCM treatment raises the possibility that GJA1-20k acts as a structural or regulatory factor in this process, similar to its role in stress-induced mitochondrial fission in cardiomyocytes [39]. In prostate cancer, mitochondrial DRP1-mediated fission is necessary to maintain the plasticity and self-renewal of cancer stem cells

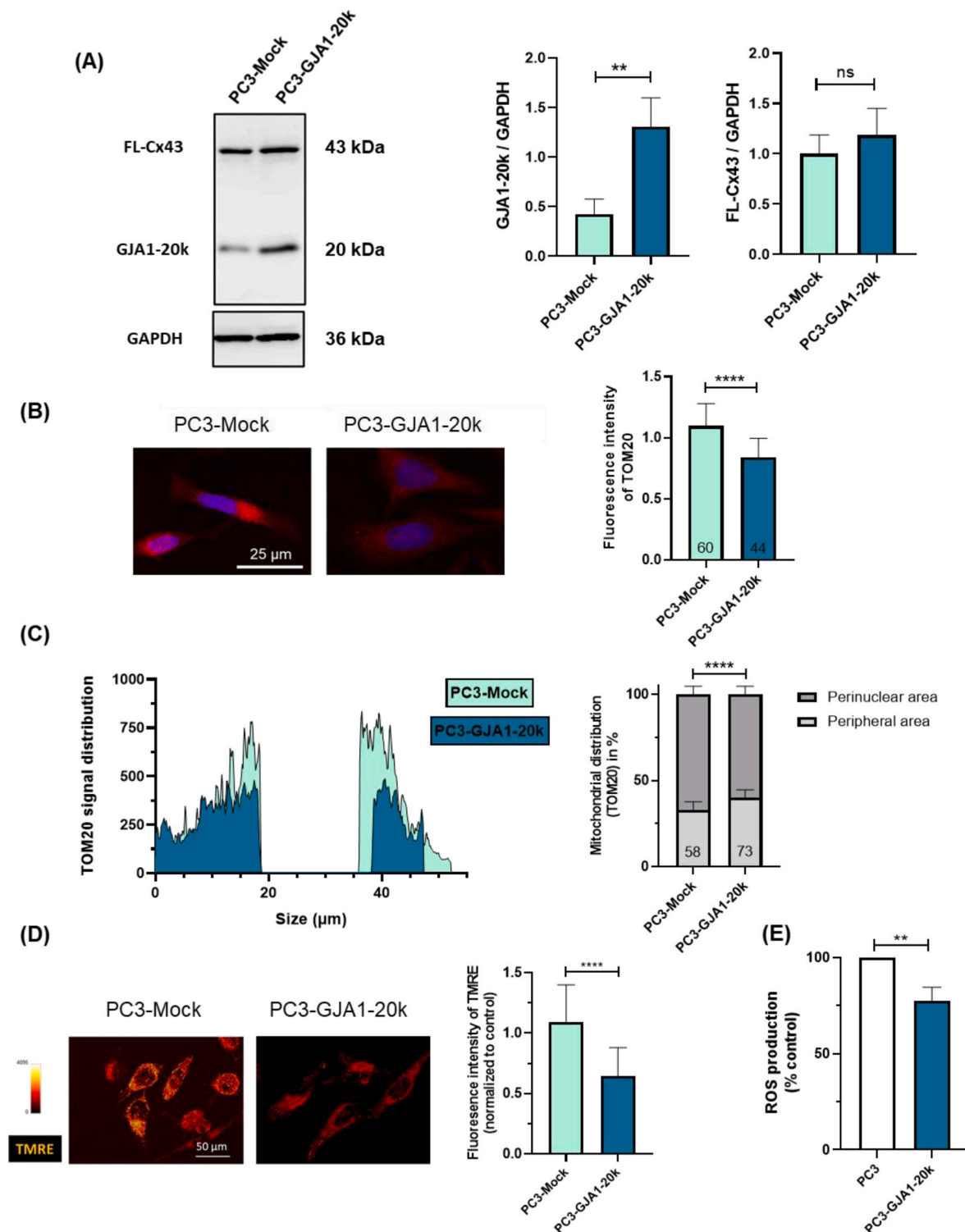


Fig. 5. GJA1-20k Overexpression Drives Mitochondrial Network Remodeling in Metastatic Prostate Cancer Cells.

(A) Representative immunoblot and densitometric quantification of GJA1-20k and FL-Cx43 expressions in GJA1-20k-overexpressing PC-3 cells (PC3-GJA1-20k) compared to control cells (PC3-mock). GAPDH was used as loading control (N = 3, each performed in duplicate). (B) Representative images of mitochondrial network and quantification of mitochondria using TOM20 immunostaining in PC3-GJA1-20k and PC3-mock cells. (C) Analysis of mitochondrial distribution in peripheral and perinuclear regions in PC3-GJA1-20k and PC3-mock cells using TOM20 immunostaining. Scale bar = 25 μ m (N = 3). Numbers inside the bars indicate the total number of analyzed cells. (D) Representative images of mitochondrial activity, measured by TMRE fluorescence intensity, in PC3-mock and PC3-GJA1-20k cells, with quantification (N = 3). Scale bar, 50 μ m. (E) Intracellular ROS production in PC3-mock and PC3-GJA1-20k cells after 48 h of culture (N = 4). *p < 0.05 to ****p < 0.0001.

(CSCs) since its inhibition led to the loss of their tumorigenic potential [40]. GJA1-20K could then participate in a compensatory mechanism that preserves asymmetric division and stemness of CSCs if this canonical DRP1-dependent pathway is impaired. Under bone stress conditions, it would also be interesting to evaluate in PCa cells the effect of GJA1-20k on mitochondrial metabolism as well as on the shape of mitochondria known to change in order to adapt to hypoxia-reoxygenation cycle [41].

Beyond its effect on mitochondria, GJA1-20k also functions as a capping protein that promotes actin filament formation [11,39]. Stabilization of actin filaments by GJA1-20k contributes to microtubule orientation toward cellular junctions, a crucial step for proper Cx43 delivery to the plasma membrane as demonstrated *in vitro* and *in vivo* [9, 10,42]. As previously demonstrated [8,43], the adhesion capacity of PCa cells to osteoblasts increases in the presence of membranous Cx43, and this effect may be enhanced by GJA1-20k overexpression, particularly under stress conditions. Moreover, once full-length Cx43 is exported to the membrane, gap junction-mediated communication facilitates calcium transfer from osteogenic cells to cancer cells, enhancing metastatic potential [4]. Finally, based on previous studies demonstrating that GJA1-20k regulates actin dynamics, mitochondrial tethering to microtubules, and Cx43 membrane trafficking [9,11,12], it is tempting to speculate that GJA1-20k could also contribute to intercellular mitochondrial transfer within the bone metastatic niche, potentially through tunneling nanotubes (TNTs). This hypothesis is consistent with reports describing stress-induced TNT formation between prostate cancer cells and osteoblasts [44], as well as evidence from other cellular models showing GJA1-20k/Cx43-dependent mitochondrial transfer via TNTs from astrocytes to neurons [45]. However, this possibility was not addressed in the present study and remains to be experimentally tested.

5. Conclusion

Our study identifies GJA1-20k, a truncated isoform of Cx43 produced through IRES-mediated translation, as a novel regulator of mitochondrial distribution in metastatic PCa cells under bone stress conditions. This highlights the role of stress-responsive, non-canonical translation in helping cancer cells adapt to the bone metastatic microenvironment [46]. By demonstrating the involvement of GJA1-20k to mitochondrial remodeling, our work suggests that cap-independent translation supports the production of stress-responsive proteins and contributes to metabolic adaptation in prostate cancer cells exposed to bone-like conditions. These insights complement previous reports highlighting the importance of hypoxia-driven translational reprogramming in prostate cancer progression and therapy resistance [47], and point to GJA1-20k as a potential target for disrupting the adaptive strategies that enable metastatic persistence. Further research into how GJA1-20k is regulated and how it functions in this context could help clarify its role in cancer cell dormancy and reactivation, and may lead to new strategies to limit disease progression in patients with bone metastases.

Funding sources

This research was supported by grants from the “Ligue Nationale contre le Cancer” (comités des Deux-Sèvres et de Charente) and from the University of Poitiers. S. Fournier is the recipient of a doctoral fellowship from the “Ministère de l'enseignement supérieur et de la recherche”.

CRediT authorship contribution statement

Sarah Fournier: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Nicolas Delannoye:** Data curation, Investigation, Methodology, Validation. **Isabelle Courtin:** Methodology, Resources. **Thomas Harnois:** Data curation, Investigation, Methodology, Validation. **Anne Cantereau:** Data curation, Investigation, Methodology, Validation,

Writing – review & editing. **Jonathan Clarhaut:** Data curation, Investigation, Methodology, Validation, Writing – review & editing. **Clarisse Vandebrouck:** Data curation, Investigation, Methodology, Writing – review & editing. **Laurent Cronier:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Arnaud Monvoisin:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work benefited from the expertise and facilities of ImageUP, the microscopy platform of the University of Poitiers. We thank the Région Nouvelle-Aquitaine and the European Union for their equipment grant programs supporting research laboratories and platforms.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2026.154336>.

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