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Review Article

Studies of hormonal regulation, phenotype plasticity, bone metastasis, and experimental therapeutics in androgen-repressed human prostate cancer (ARCaP) model

U-Ging Lo[1*], Yu-An Chen[1*], Zahraa I Khamis[2,3*], Wei-Hsiang Kao[1], Jer-Tsong Hsieh[1], Qing-Xiang Amy Sang[2]

[1]Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA; [2]Department of Chemistry & Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306, USA; [3]Department of Chemistry and Biochemistry, Faculty of Sciences-I, Lebanese University, Beirut 999095, Lebanon. [*]Equal contributors.

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Abstract: First established by Dr. Leland W. K. Chung's lab, the androgen-repressed prostate cancer cell (ARCaP) line is derived from the ascitic fluid of a prostate cancer (PCa) patient with widely metastatic disease. Based on its unique characteristic of growth suppression in the presence of androgen, ARCaP cell line has contributed to the research of PCa disease progression toward therapy- and castration-resistant PCa (t-CRPC). It has been widely applied in studies exploring experimental therapeutic reagents including Genistein, Vorinostat and Silibinin. ARCaP cells have showed increased metastatic potential to the bone and soft tissues. In addition, accumulating studies using ARCaP model have demonstrated the epithelial-to-mesenchymal transitional plasticity of PCa using epithelial-like $ARCaP_E$ line treated *in vitro* with growth factors derived from bone microenvironment. The resulting mesenchymal-like $ARCaP_M$ sub-clone derived from bone-metastasized tumor has high expression of several factors correlated with cancer metastasis, such as N-Cadherin, Vimentin, MCM3, Granzyme B, β2-microglobulin and RANKL. In particular, the increased secretion of RANKL in $ARCaP_M$ further facilitates its capacity of inducing osteoclastogenesis at the bone microenvironment, leading to bone resorption and tumor colonization. Meanwhile, sphingosine kinase 1 (SphK1) acts as a key molecule driver in the neuroendocrine differentiation (NED) of ARCaP sublines, suggesting the unique facet of ARCaP cells for insightful studies in more malignant neuroendocrine prostate cancer (NEPC). Overall, the establishment of ARCaP line has provided a valuable model to explore the mechanisms underlying PCa progression toward metastatic t-CRPC. In this review, we will focus on the contribution of ARCaP model in PCa research covering hormone receptor activity, skeletal metastasis, plasticity of epithelial-to-mesenchymal transition (EMT) and application of therapeutic strategies.

Keywords: ARCaP, receptor activator for nuclear factor κB ligand (RANKL), NED, EMT, castration-resistant prostate cancer

Introduction

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer mortality in men of the United States [1]. Up to this time, androgen deprivation therapy (ADT) remains a therapeutic mainstay for metastatic PCa [2, 3]. While the onset of castration-resistant PCa (CRPC) [4] once symbolized the end stage of the disease, the appearance of therapy- and castration-resistant CRPC (t-CRPC) exhibiting neuroendocrine (NE) phenotypes following treatment with second generation of anti-androgens, seems inevitable [5-8]. However, the mechanisms of transdifferentiation of these PCas remain unclear due to the limited availability of representative cell models.

In 1996, Drs. Haiyen E. Zhau and Leland W. K. Chung's team reported an ARCaP cell line derived from the ascites fluid of a patient with advanced PCa widely disseminated in bone and soft tissues [9]. In particular, this ARCaP line was demonstrated to be highly metastatic and tumorigenic when re-inoculated into athy-

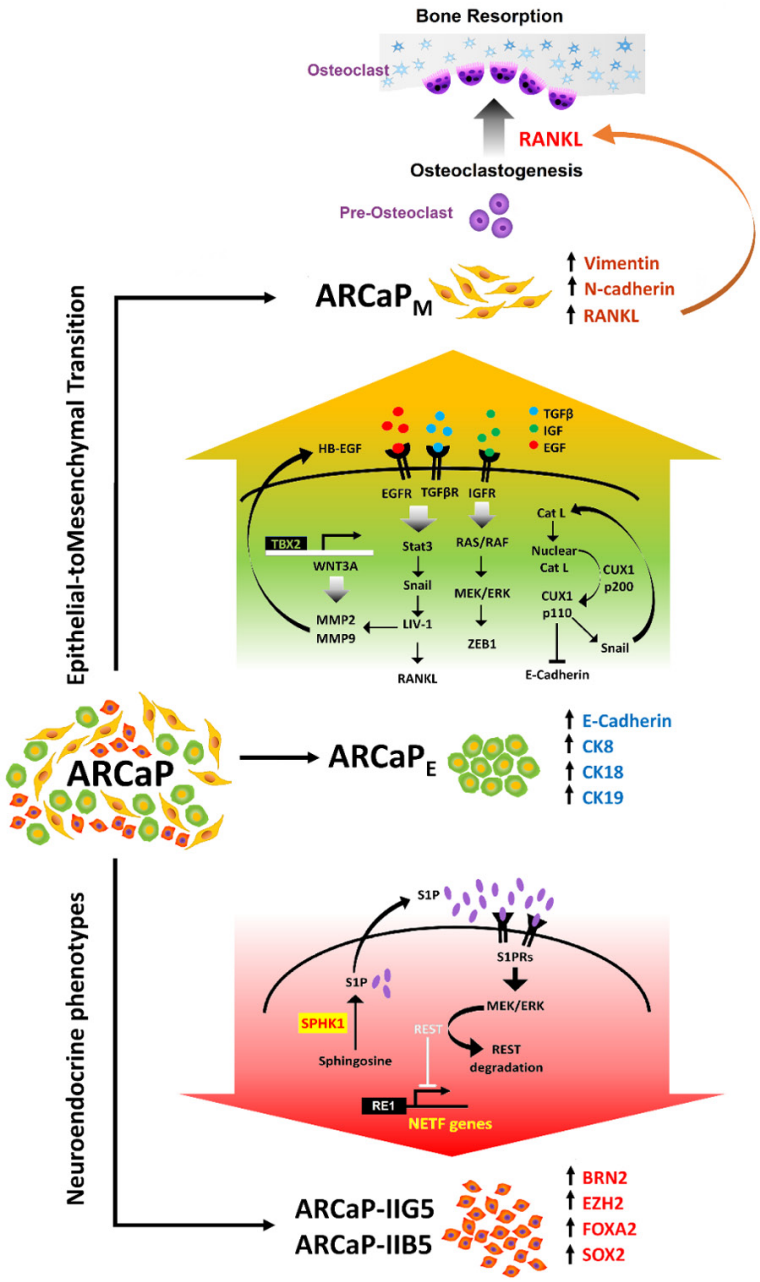

Figure 1. Molecular mechanism of prostate cancer progression to lethal phenotypes in ARCaP.

mic mouse model by either intracardiac or orthotopic transplantation [10]. Despite its low expression of both androgen receptor (AR) and prostate specific antigen (PSA), the cellular growth of ARCaP line could be suppressed by estrogen or androgen [9]. The impact of androgen on ARCaP growth may be partially due to the presence of phosphorylated androgen-induced proliferation inhibitor (APRIN) [11] that inhibits cell proliferation in the presence of androgen [12, 13]. However, no mechanistic study has been conducted to reveal the function of APRIN in ARCaP line.

Based on the unique characteristics of ARCaP as an androgen repressed PCa line, several studies using the ARCaP model were conducted that delineated the mechanisms of hormone receptor activity during disease progression from CRPC to t-CRPC [14, 15].

Furthermore, using *in vitro* growth factor induction or *in vivo* xenograft strategy, epithelial-like (ARCaP$_E$) and mesenchymal-like (ARCaP$_M$) sub-clones were established based on their cellular morphology and epithelial-to-mesenchymal transition (EMT)-related gene expression profile [16]. In particular, studies using the ARCaP$_E$ sub-clone have unveiled the mechanisms underlying the dynamic transition of PCa from epithelial to mesenchymal status [17-19]. Meanwhile, the ARCaP$_M$ sub-clone provides an optimal model for studies focused on the molecular interaction between cancer cells and the osseous microenvironment upon metastasis to bone [20-22]. Collectively, ARCaP sub-clones have provided valuable information regarding mechanism of actions that are critical for the advanced progression of PCa (**Figure 1**). In this review, we will discuss the insights of hormone receptor signaling, bone metastasis, and cell plasticity based on the ARCaP parental line and sublines.

The hormone receptor dependency of ARCaP

Several studies have demonstrated that PCa cells acquire a NE-like phenotype after long-term ADT treatment [23-25]. However, the

mechanism of ADT-induced NE differentiation (NED) of PCa is not yet fully characterized. Dayon *et al.* showed the involvement of SphK1 in NED after androgen deprivation in hormone-sensitive PCa cell lines. While short-term ADT caused SphK1 inhibition, long-term androgen withdrawal resulted in activation of SphK1 activity. This ADT-induced differential effect on SphK1 correlated with NED and progression of PCa cells to an androgen-independent state [26]. Similarly, we have demonstrated a central role of SphK1 in ADT-induced NED of ARCaP cells [27] (**Figure 1**).

In addition to androgens, estrogens are involved in PCa development and progression. Estrogens were used for metastatic PCa treatment [28, 29]. Several studies have demonstrated that normal human prostate gland epithelial [30-32] and stem/early progenitor cells express estrogen receptors (ER) (ER-α and ER-β) [33, 34]. Ekman *et al.* showed that ER content was markedly lower in lymph node metastases compared with primary PCa [35]. Nativ *et al.* observed that the mean of ER expression was decreased with stage progression (T1, T2, T3 and N+) [36]. In addition, Ye *et al.* found that the expression of different ER-α variants varied substantially among ARCaP, LNCaP, and C4-2 cell lines due to their distinct androgen dependency and tumorigenic and metastatic potentials [14]. The ER-α variants such as ER-αC, ER-αD, and ER-αE were detected in ARCaP cells and might be responsible for their increased metastatic potential to bone [9]. Also, the relative ratio of the wild-type ER-α (wt ER-α) to ER-α variants in ARCaP cells is much lower than LNCaP and C4-2 cells, suggesting that the wt ER-α and increased ER-α variants in ARCaP cells may affect the differential actions of estrogen in PCa cell lines [14]. Additionally, Ye *et al.* also found that ectopic expression of wt ER- α in invasive ARCaP cell line suppressed their growth and might be involved in G0/G1 cell cycle arrest [15].

ARCaP cells metastasis to bone

Skeletal metastasis represents a serious problem in advanced PCa. Approximately 90% of men with metastatic disease suffer from bone complications. This tropism to bone is orchestrated by the tumor cells, and the bone cells and microenvironment [37, 38]. While PCa bone metastases are mainly osteoblastic, increased osteoclastic lesion markers in serum and urine are also observed [39]. The osteoblastic metastases of PCa are induced by secretion of factors that stimulate osteoblast proliferation and differentiation. In turn, tumor-induced osteoblasts and bone microenvironment are shown to promote PCa progression. Several studies have demonstrated that osteoblasts in co-culture models [40] and bone-derived stromal cells supported PCa proliferation and survival [41, 42]. The osteoclastic metastases of PCa involve the RANKL/RANK/OPG signaling pathway. RANKL (Receptor activator for nuclear factor κB ligand) is a member of the tumor necrosis factor (TNF) family of cytokines and is expressed by osteoblasts, tumor cells, stromal cells, and T lymphocytes. It exists as a transmembrane and a soluble protein. Both forms are active and stimulate bone resorption upon binding to their receptor, RANK, present on osteoclasts. Osteoclastogenesis is regulated by OPG (osteoprotegerin), a decoy receptor for RANKL that is secreted by osteoblasts. Interaction of OPG with RANKL blocks RANKL/RANK signaling and consequently inhibits osteoclast development and function [43]. Osteolytic bone metastases associated with PCa involve secretion of osteoclastic factors from tumor cells such as parathyroid hormone-related protein (PTHrP). These factors stimulate the formation of RANKL by osteoblasts and stromal cells inducing osteoclasts development. In turn, tumor-induced bone resorption releases growth factors such as TGF-β, which will stimulate tumor cells growth and further secretion of osteolytic factors resulting in a “vicious cycle” of bone resorption [38]. Once metastasized to the bone, ARCaP cells induce osteoblastic as well as osteoclastic lesions with paraplegia development [9, 44]. ARCaP subclones, $ARCaP_E$ and $ARCaP_M$ generated by EMT, induced tumor growth and skeletal metastasis with osteoblastic and osteolytic responses upon intracardiac injection in athymic mice. The mesenchymal PCa cell, $ARCaP_M$, showed increased metastatic potential to the bone when compared to the epithelial subclone, $ARCaP_E$ [10]. As expected, $ARCaP_M$ cells express more RANKL than $ARCaP_E$ cells [20]. The expression of RANKL by $ARCaP_M$ accounts for the elevated osteoclastogenesis and the subsequent bone turnover [10, 16]. Also, increased bone-

marrow adipocity mediated by increased CXCL1/CXCL2 chemokines and their receptor CXCR2 contributed to $ARCaP_M$ driven osteolysis of the bone. This was further evidenced by the increased expression of bone remodeling genes, calcineurin, cathepsin K, and MMP-9, that contributed to osteoclast differentiation [45]. Moreover, TGFβ1 and EGF co-treatment was able to stimulate RANKL expression on $ARCaP_E$ cell promoting osteoclastogenesis [20] (**Figure 1**).

Role of tumor microenvironment in ARCaP cells skeletal metastasis

The predilection of PCa cells to metastasize to the bone results from the reciprocal interaction between the tumor cells and surrounding microenvironment [46, 47]. This interaction can occur through soluble factors or matrix molecules which will cause the co-evolution of both tumor and stromal cells into more invasive and inductive phenotypes respectively [48]. To metastasize to the bone, PCa cells need to gain access to bone tissue, adapt to its microenvironment while being dormant, proliferate, and finally transform bone structure and function to support their growth. Each step of the metastatic process involves direct communication between bone microenvironment and PCa cells (reviewed extensively by Zhang X. 2019) [49]. So, co-targeting both cancer cells and surrounding stromal cells proved to be effective. Using a conditional replication-competent adenovirus, the growth of ARCaP cells and other PCa cell lines and osteoblasts was blocked [50]. The bone microenvironment modulated the efficacy of cabozantinib, a tyrosine kinase inhibitor, in PCa bone metastasis. Cabozantinib was much more potent on host macrophages and tumor associated and bone marrow fibroblasts than on prostate epithelial cancer cells. Cabozantinib reduced the number of M1 macrophage without effecting the M2 macrophage when compared with the control mice. Furthermore, the drug affected monocyte differentiation and reprogrammed the tumor microenvironment to be pro-tumorigenic [51].

The importance of tumor cell-bone microenvironment interplay in PCa cell tropism to bone is further validated using conditioned medium from ARCaP cells and bone stromal cells. The conditioned medium was found to enhance the expression of the bone-specific proteins, osteocalcin and bone sialoprotein, by PCa cells [52]. These bone matrix proteins are synthesized by osteoblasts and are also expressed by PCa cells, which may explain the ability of cancer cells to adhere and grow in the bone microenvironment, and to mimic bone cells [53]. The osteomimetic phenotype of PCa cells is regulated by secretion of β2-microglobulin, which through activation of cAMP-dependent PKA pathway, and cAMP responsive element binding (CREB) and its bone-associated target genes confers tumor growth and colonization in bone [53].

The epithelial subclone of ARCaP human PCa cells, $ARCaP_E$, has low bone metastatic potential when inoculated intracardiacally, but shows 100% metastasis to bone when derived from bone metastases [10]. The bone-derived cells have distinct morphology, share mesenchymal cells gene expression profile like N-cadherin, RANKL, and IL-13 receptor α2, and thus are termed $ARCaP_M$ [16, 20]. Additionally, *in vitro* exposure of $ARCaP_E$ cells to soluble growth factors such as TGFβ1 plus EGF, IGF-1, Snail and Slug transcription factors, β2-microglobulin induces the switch into the mesenchymal phenotype, $ARCaP_M$ [10, 16, 20]. Specifically, overexpression of β2-microglobulin induces EMT and PCa proliferation, invasion, and bone metastasis [54]. Together, the transition of $ARCaP_E$ into $ARCaP_M$ is triggered by bone microenvironment (**Figure 1**).

The plasticity of epithelial to mesenchymal transition in ARCaP sublines

From the morphologically distinctive ARCaP sublines, spindle-shaped $ARCaP_M$ exhibits increased Vimentin expression as well as decreased E-cadherin, CK18 and CK19 expression compared with the cobblestone-shaped $ARCaP_E$ (**Figure 1**). As expected, $ARCaP_M$ has a significantly higher cell migration and invasion potential *in vitro* and has 100% incidence of bone metastasis *in vivo* compared to 12.5% incidence rate in $ARCaP_E$. $ARCaP_M$ showed higher expression of the mini-chromosome maintenance 3 (MCM3) protein than $ARCaP_E$, a subunit of DNA helicase, which was also found to be significantly elevated in ARCaP-derived bone metastasis and in more advanced PCa tissues [55]. Based on the fact that ARCaP subclones, $ARCaP_M$ and $ARCaP_{AG}$, derived from bone and adrenal gland metastatic sites respectively have distinct morphology and gene expression profiles suggests that the host microenvironment is a major trigger in

EMT and dictates the phenotype of the trans-differentiated ARCaP cells [10].

Additionally, Bou-Dargham *et al.* examined the secretory proteins released from $ARCaP_E$ and $ARCaP_M$ by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and discovered that Granzyme B (GZMB) was highly secreted by the more aggressive $ARCaP_M$ cells. In concordance, loss of GZMB gene significantly diminished the invasiveness of $ARCaP_M$ line, suggesting a novel role of the extracellular GZMB in promoting cancer cell invasion [56].

Moreover, PMA (Phorbol ester phorbol-12-myristate-13-acetate), a potent PKC antagonist, was found to induce EMT in $ARCaP_E$, and to increase their migratory and invasive potentials [57]. Meanwhile, Zhau *et al.* also demonstrated the dynamic plasticity of PCa by exposing $ARCaP_E$ to several soluble growth factors (TGFβ, EGF, IGF-1, or β2-microglobulin) *in vitro* or to bone microenvironment *in vivo* [16]. In parallel to Zhau's study, Graham also used $ARCaP_E$ and $ARCaP_M$ subclones to demonstrate that the highly expressed ZEB1 in $ARCaP_M$ is not only correlated with disease progression of PCa, but also critical for the mesenchymal transition of advanced PCa [17]. Additionally, Lue *et al.* showed the driver role of LIV-1 in EMT of $ARCaP_E$ cells through shedding of soluble heparin binding-epidermal growth factor (HB-EGF) by MMP2 and MMP9 and subsequent phosphorylation of EGFR leading to massive bone and soft tissue metastases [18]. Furthermore, the transcription factor, Snail, was found to induce the expression of urokinase-type plasminogen activator (uPA) and its receptor uPAR as well as integrins (α5, α2, and β1) mediating ARCaP invasion [58, 59]. Other studies also demonstrated that ARCaP invasiveness is powered via the production and activation of matrix metalloproteinases (MMPs). ARCaP cells produced a large amount of MMPs including activated MMP-2, MMP-3, MMP-9, and MMP-26, which together enhanced cell invasion and metastasis [9, 60]. Overall, this series of ARCaP sublines provides a valuable model for researchers to study EMT and lineage plasticity during PCa progression.

Exploration of experimental therapy on therapy resistant ARCaP

ARCaP cells are derived from a patient who has failed to respond to hormonal therapy, which mimics t-CRPC. Therefore, ARCaP model has been commonly used for exploring experimental therapy. Zhang *et al.* demonstrated that low dose Genistein (4',5,7-trihydroxyisoflavone, 0.5-1.0 μM), a chemo-preventive agent, exhibits significant inhibition of cell invasiveness and EMT using single cell clone of ARCaP (IA8-ARCaP) model [61]. A recent study [62] demonstrated that Genistein can increase Histone, H3K9, acetylation on the promoter region of several Wnt inhibitor genes including SOX7, APC, DKK3, WIF1, SFRP1, and SFRP2 in ARCaP sublines. Hence, the combination of Genistein with Histone deacetylase (HDAC) inhibitor, Vorinostat, leads to a significant increase in cell apoptosis in both $ARCaP_E$ and $ARCaP_M$ lines. This observation is evidenced by a whole genome profiling of Genistein or Vorinostat treated ARCaP lines. In particular, combination treatment of both drugs in $ARCaP_E$ and $ARCaP_M$ leads to enrichment of genes involved in DNA damage, cell cycle arrest, and apoptosis. However, $ARCaP_M$ cells become less sensitive to Genistein or Vorinostat-induced cell death when compared to $ARCaP_E$, which could be due to downregulation of several anti-apoptotic genes such as BIRC7/Livin, TGFB1I1/ARA55, HES1, and SLUG in $ARCaP_M$ [62].

Meanwhile, Wu *et al.* observed that Silibinin, a naturally occurring flavanone isolated from milk thistle extract, exhibited a strong anticancer effect evidenced by inhibition of $ARCaP_M$ cell migration and invasion via downregulation of Vimentin and MMP2 [63]. In a recent study, Burton *et al.* demonstrated that the treatment with the antioxidant Muscardine grape seed extract in ARCaP model can abrogate Snail-mediated superoxide species and decrease cell migratory capacity via antagonizing JAK-Stat3 signaling pathway [64].

Conclusions

Professor Leland W. K. Chung's research team has made immense contributions to PCa research field, especially for establishing metastatic human PCa cell line models, including CRPC ARCaP sublines (**Figure 2**). The discovery of ARCaP cells was quite accidental. It began with a search for soluble factors that regulate prostate-specific antigen from the ascites fluid of a patient with metastatic prostate cancer. After the clear supernatant was

Figure 2. Professor Leland W. K. Chung in his office on December 15, 2017. Dr. Chung was the director of the Urologic Oncology Research and a Professor in the Departments of Medicine and Surgery Program at Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California. Dr. Chung has left a great scientific legacy as a leader, innovator, mentor, role model, scientist, educator, and friend to many of us. He has made tremendous impact on generations of scientists and on human prostate cancer research field. He is dearly missed. (Photo Courtesy of Dr. Haiyen E. Zhau).

collected, a cell biologist in the laboratory discarded the centrifuge tubes to the sink to be washed. Drs. Leland W. K. Chung and Haiyen E. Zhau happened to be standing by the sink at the moment. Curious if there was any sign of cell life, Dr. Chung picked up the tubes and delivered them to the cell biologist to lay some debris and pellets in culture media. This was the birth of ARCaP cells. At the time, it was such an unexpected discovery of cells from recovered discarded waste. A series of *in vitro* and *in vivo* characterization of the cells was carried out. Amazingly, ARCaP cells exhibited an unusual androgen-repressed response and were positive for several neuroendocrine factors. The ARCaP cells became known in the lab as ET (or Extra-Terrestrial, after the popular movie). In the first ARCaP publication on PNAS, the entire study was carried out using the original ARCaP cells as mixed clones [9]. Further single-cell cloning of ARCaP cells produced various cells with distinct morphological and biochemical properties. Not all the single cell clones are androgen repressed. Since then, androgen-refractory cancer of the prostate for ARCaP has been used. These ARCaP sublines provide PCa research models for hormonal regulation, bone metastasis, neuroendocrine differentiation, cancer cell phenotype plasticity, epithelial-to-mesenchymal transition, tumor-microenvironment interactions, and evaluation of experimental therapeutics. These cell line models will enable further discovery of molecular profiles and biochemical mechanisms of human PCa progression and metastasis, thus, facilitating drug discovery and curing PCa. Dr. Chung will be greatly missed by all of us and generations of cancer research to come, and his legacy and love for science and humanity will be eternal (**Figure 2**).

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Disclosure of conflict of interest

None.

Address correspondence to: Jer-Tsong Hsieh, Department of Urology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA. E-mail: JT.Hsieh@UTSouthwestern.edu; Qing-Xiang Amy Sang, Department of Chemistry & Biochemistry and Institute of Molecular Biophysics, Florida State University, 600 W College Avenue, Tallahassee, FL 32306, USA. E-mail: qxsang@chem.fsu.edu

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