

REGULATION OF PROSTATE SPHERE FORMATION POTENTIAL BY NIH 3T3
FIBROBLAST CELLS

by

ZIXUAN ZHEN

(Under the Direction of Houjian Cai)

ABSTRACT

Stromal-epithelial interaction is essential for maintaining the niche of stem cells and regulating organ development and cancer progression. Numerous cell signaling pathways facilitate stromal-epithelial interaction. Among those, androgen receptor (AR) signaling in fibroblast cells regulates prostate stem cells (PrSCs), thereby controlling prostate development under the induction of endogenous androgen and exogenous inhibitors. In this study, we used an *in vitro* experiment to study biological function of NIH 3T3 fibroblasts and molecular function of AR in regulating the sphere formation potential of prostate primary cells. We demonstrate that co-culture of 3T3 fibroblast cells with prostate primary cells significantly increased the sphere number. Additionally, overexpression of AR in 3T3 cells further enhanced sphere formation potential. However, activation of AR signaling by addition of an AR agonist R1881 inhibited the sphere number. Our study suggests the functional role of fibroblast cells and the goldilocks' effect of AR signaling in regulation of PrSCs activity.

INDEX WORDS: Androgen receptor; prostate stem cell; prostate sphere; prostate stromal cell; R1881; co-culture of stromal cells and sphere.

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CHAPTER 1

INTRODUCTION

The structure of prostate

As the largest accessory gland in the male reproductive system, the prostate has the main function of storing and secreting prostatic fluid contributing to micturition and ejaculation. Moreover, prostate plays a physically important role in male health and fertilizable enhancement as well [1].

The prostate is an unpaired substantial gland which posits between the bladder and the muscle of urogenital diaphragm and wraps the root of the urethra [2]. It can be touched by a digital rectal exam since it is located in front of the rectum. Normally, the prostate is a chestnut-sized gland with the weight about 6-20 grams and the mean weight is about 11 grams. The average volume is about 20 cm³, which can be predicted well by the formula: $0.52 \times \text{length} \times \text{width} \times \text{height}$ [3].

From a microscopic point of view, prostate tissue is composed of the epithelial gland and stroma, in which approximately two-thirds of it is glandular in structure while the remaining third is fibromuscular in structure [4]. The gland is wrapped by a thin fibrous capsule of the prostate [5], and its inner layer is formed by the tall column-shaped cells which lie in either one layer or are pseudostratified [6]. The appearance of epithelium is highly variable, including but not limit to the low cuboidal, squamous epithelium areas or the transitional epithelium at the distal ends of the longer ducts [4]. Many follicles are included in the glands, which are connected with long canals, followed by 12 to 20 main ducts, which flow through the prostate and subsequently drain into the urethra [6]. The size of basal cells is small and they locate next to a thin, fibrous, extracellular matrix called basement membranes of glands. The stroma of the prostate is composed of fibrous

tissue and smooth muscle. And the gland can be divided into several subdivision, lobules, which are usually called in anatomy, by the fibrous tissue [4]. Then the prostate can be subdivide into five lobes, including anterior lobe, posterior lobe, right lateral lobe, left lateral lobe and median lobe or called middle lobe in mice [2].

The mature prostate involves two different cell types, epithelial cell, and stromal cell [3]. The epithelial cells could be subdivided into several cell types, which appears in quite different morphology in the mature prostate, including basal cells, luminal cells, neuroendocrine cells which are rare [1]. With morphology of tall columnar like, the luminal cells are epithelial cells with expression of cytokeratin (CK) 8 and 18, CD 24 and 26 surface protein as well as a various of secretory proteins such as prostate specific antigen (PSA) [7]. As for the basal cells compartment, they are non-secretory cells lining the basement membrane and below the luminal layer expressing CK5, CK14, CD44 and p63, while they lack PSA expression[7, 8]. According to El-Alfy and Mirosevich's research, expressing levels of androgen receptor (AR) in the prostate basal cells in mouse and human is low or undetectable, while nearly all of luminal cells could express high levels of AR [9, 10]. There are also intermediate cells lie within the basal layer which are luminal cells differentiated from basal cells. The intermediate cells express markers found in both luminal and basal cells as well as additional markers such as CK19 [11]. In spite of that, it still remains unclear whether intermediate cells behave as an individual cell subtype with other functions. Finally, rare neuroendocrine cells expressing secreted neuropeptides and other hormones disperse among the basal cells as well, and often act a role in a dendritic-like process that contacts the glandular lumen [12].

In addition, the mesenchymal compartment in the prostate is also made up by a variety of differentiated cell types, whereas the stroma layer surrounding the epithelial cells is consist of

smooth muscle cells and fibroblasts. For example, a layer of smooth muscle cells is formed by the cells of the embryonic urogenital sinus mesenchyme (UGM), which frame in the epithelium and control the expulsion of prostatic fluid in the process of ejaculate via their contractile activity [13]. While, a large population of mature fibroblasts are included in the adult prostate stroma and secrete various components such as glycoproteins, fibrillar proteins, and proteoglycans to form the extracellular matrix. On basis of the support, a structural network is made up allowing the mediate of growth factor signals [14]. Finally, other common components including nerves, blood vessels, lymphatics, and immune cells in the stroma are also essential for the regulation and development of the prostate. The structural features of prostate are shown in Figure 1.

The development of prostate

Prostatic development has been widely studied and investigated in numerous animal models and a few of human fetal models. Androgenic stimulation takes an unquestionable role in the development of prostate. In concept, there are basically four stages in the prostate organogenesis process [15].

In the first stage, the prostate is initially raised from a primitive urogenital sinus (UGS), which is divided from the caudal extension of the hindgut during the embryogenesis occurring at 8 weeks of gestation in humans and 13.5 days post coitum (dpc) in the mouse [16, 17]. The UGS could form functional prostatic tissue mediated by androgens no matter it comes from male or female fetuses which lead to the prostate induction in males [18]. At approximately 10 weeks of gestation in humans and 17.5 dpc in mouse, the prostate is comprised from the epithelial budding of UGS [19]. Next, under the initiation of prenatal androgenic action, the urogenital sinus epithelium (UGE) surrounded by urogenital sinus mesenchyme (UGM) buds into the UGM, initiating the

process of tissue outgrowth, canalization, epithelial cytodifferentiation and ductal branching morphogenesis to form a system of ducts composed of solid epithelial cords. In this evolution, the function of androgen receptor (AR) is significant to mediate epithelial outgrowth in paracrine signaling from the UGM to the UGE [20]. Subsequently, the outgrowth of ductal tissue triggers the process of ductal branching morphogenesis, then induces the mature ductal network. This process gives rise to the formation of five different prostatic lobes in the mouse as mentioned previously. However, this process in humans results in the formation of distinct prostatic zones within a uni-lobular prostate organ. Finally, the solid epithelial cords go through canalization to form the ductal lumen as well as cytodifferentiation to induce the functional glandular epithelium with terminally differentiated cell types [1, 15].

Stromal-epithelial interaction

Cell-cell interaction is believed to play a vital role in prostate development, growth, and differentiation. In the embryonic period, the undifferentiated connective tissue that called mesenchyme has interaction with epithelial cells which plays an utmost important role in the embryonic prostate development. The mesenchyme induces the epithelial cells development at the same time the epithelium induces the differentiation of mesenchymal and the pattern-forming of the morphology of smooth muscle in the UGS [21]. Studies have shown that, influenced by the present of androgen, urogenital sinus mesenchyme (UGM) could induce urogenital sinus epithelium to form prostatic ductal morphogenesis and undergo differentiation. After the prostatic epithelium differentiates, it will signals the UGM in turn. Under the influence of epithelial cells, the UGM differentiates into smooth muscle cells which are surround the prostatic epithelial ducts [22]. Once the prostate formed, prostatic stromal compartment interacts with epithelial cells in

reciprocal. Differentiation of UGM to prostatic smooth muscle requires not only the inductive signal from epithelium but also androgens.

Mesenchymal-epithelial interaction is devoted to describing the cell-cell interaction in the embryonic stage, while stromal-epithelial interaction is applied to that in the adult period [23]. In adulthood, stromal-epithelial cell interaction regulates normal epithelial growth and differentiation through local synthesis and action of paracrine signaling molecules. There exist reciprocal signaling between mouse prostate epithelial and stromal progenitor cells. Mouse prostate stromal cells signal to epithelium through Wnt ligands, fibroblast growth factors (FGFs), and transforming growth factor- β (TGF- β). Mouse prostate basal cells signal to a number of stromal cell-types through Sonic hedgehog[24]. Studies have demonstrated that the prostatic stromal-epithelial interaction is of importance for mediating the effect of steroid sex hormones such as testosterone, dihydrotestosterone which is critical for the epithelial proliferation, differentiation and protein secretion of the prostate gland [25]. Under the help of androgen, the stromal cells aid for holding the epithelial structure and function. In turn, the prostatic epithelium can help stromal differentiation [26].

Prostate stem cell

Tissue-specific murine stem cells are undifferentiated cells with capacity for long-lasting self-renewal and differentiation into mature progeny cells at a single cell level. The mature progeny cells include two subtype, the non-renewing progenitors and terminally-differentiated effector cells that constitute distinct cell types within the tissue of interest [27]. The discovery of a stem cell type in the prostate can be traced back to 1980s [28]. In epithelial cells from adult prostate, basal cells and luminal cells are two lineages that are sustained to themselves, rare basal-to-luminal

differentiation occurred [29, 30]. Researchers have found that the prostate gland can undergo multiple rounds of regression and regeneration. They demonstrated that the rodent prostate significantly reduced after surgical castration or androgen removing because of the apoptosis of luminal cells [31], and then increased proliferation after testosterone propionate injection [32]. The hypothesis has come out based on this observation that a population prostate stem cells should resist to castration [33, 34].

As for the location of the prostate stem cell, there are numerous evidences of distinct phenotype of the prostate stem cells with either basal or luminal cells features. According to the individual molecules on the cell surface, the prostate cells can be classified into specific cell types. For examples, prostate stem cells express stem cell antigen-1 (Sca-1). While basal cells are CD49f positive, luminal cells are CD24⁺ and CD49f⁻. Additionally, stromal cells are usually CD34⁺. Some hematopoietic and endothelial cells are also part of prostate tissue. They are both CD45 and Ter119 positive, and CD31 positive cell lineages (Lin⁻), respectively. Cells with the capacity of self-renewal show Sca-1⁺CD49f⁺Lin⁻ both in vitro. The same markers are expressed in prostatic ducts containing basal and luminal cells, but these markers could be used to discriminate the prostate stem cells from differentiated epithelial cells [35]. Additionally, single Lin⁻Sca-1⁺CD133⁺CD44⁺CD117⁺ cells are capable of generating prostate tissue when transplanted with embryonic urogenital sinus mesenchyme(UGSM) cells in renal grafts [36]. The regenerated tissue possess secretion-capability. These evidences support the idea that prostate stem cells locate at the basal layer of the prostate gland.

On the contrary, Kurita found that explants in p63-null mice which lack basal cells could form prostatic ducts which contain both basal cells and luminal cells when induce UGSM cells and testosterone [37]. These data suggest that a distinct group of prostate stem cell exist in the luminal

layer. Additionally, prostate cancer cells show luminal cells phenotype and basal cells are absent in prostate carcinoma. Prostate cancer is believed to be proliferation of luminal cells and expansion of basal cells in prostate cancer is rare. These suggest that prostate cancer origins from luminal cells, and is transformed by oncogenic events, which lead to differentiation of basal stem cells into luminal cells [38]. Based on Xi's research, the homeobox gene Nkx3.1 is a marker of stem cell population with the function of prostate regeneration. Genetic lineage-marking indicates that rare luminal cells expressing Nkx3.1 can generate basal and luminal cells in the absence of testicular androgens (castration-resistant Nkx3.1-expressing cells, CARNs), suggesting the bipotent nature of the CARNs. Also CARNs can self-renew in vivo as well as reconstitute prostate ducts in kidney capsule based on single-cell transplantation assays [39]. These demonstrate that the existence of PSCs could be luminal cell origin.

Prostate sphere

Murine prostate stem cells express integrin $\alpha 6$, which regulate proliferation and differentiation when interacting with laminin. When plating the prostate stem cells in the Matrigel, which contain the laminin and allow cells to grow in it, 1 out of 500-1000 prostate stem cells can form a clonogenic spheroid structure. Based on the previous study, although dissociated prostate primary cells contains epithelial cells, stromal cells and other cells, only epithelial stem cells can grow into spheres [35]. Other stromal cells isolated from the prostate are not able to form a sphere. Additionally, it has been tested that 3T3 alone will not form sphere when growing in the matrigel. The prostate sphere is defined as clonal spheroids that formed from murine prostate cells. Matrigel is comprised primarily of laminin, collagen, fibronectin and other extracellular matrix (ECM) components. This in vitro environment closely mimics the ECM- rich basement membrane where

basal cells reside in the gland. The prostate stem cells quickly lose their self-renewal potential when they are cultured in two-dimensions [40]. Therefore, a three-dimensional culture system maintain and expand primitive prostate cells that retain the capacity for self-renewal [41].

The sphere cells express CD24, CD49f, and Sca-1 uniformly and all lack CD90, CD133, and CD44 surface markers. Androgen receptor could be detected in the sphere cells via western blot but the majority of AR was partially degraded, only a molecular mass of around 87kD displays apparently [42]. The transcription factor p63 which is expressed in prostate basal cells also present in the outer layer of the prostate sphere. Ki-67 staining shows that most p63 expressing cells are able to proliferate actively, whereas terminal deoxynucleotidyl transferase dUTP nick-end labeling assay shows the presence of apoptotic cells in the cores of the spheres. These phenotype indicate that the prostate sphere cells maintain a distinct cellular hierarchy and structural organization. Also, it is discovered that sphere cells display a more differentiated transit-amplifying cell phenotype in the center while a more early stem/progenitor cell phenotype at the rim of the sphere [41].

Sphere-forming cells have self-renewal capacity [41]. When the prostate spheres are dissociated into single cells and reseed them back, the dissociated single cells can be passed to generate daughter sphere with the same composition. The cells that form the sphere also have multilineage differentiation ability due to the phenotype of prostate stem/progenitor cell. The prostate sphere-forming cells undergo further differentiation when inducing the stimulation of 5α -dihydrotestosterone. When implanted *in vivo*, a fraction of the spheres can also keep the capacity of self-renewal and multilineage differentiation, and form prostate tubular structures. The formed prostate tubules are composed of established basal and luminal layers together with prostatic secretions which could resemble prostate tissue.

As two key characteristics of the stem cells, self-renewal and multilineage differentiation have been well studied. Prostate regeneration method is used to study the differentiation capacity of the prostate stem cells [35, 43]. The self-renewal capacity has been demonstrated by castration-regeneration experiments in rodents [44]. The prostate sphere model is the first method for studying the self-renewal capacity of the prostate stem cells at a clonal level in vitro. The prostate sphere assay gives us the access for studying the in vitro maintenance and characterization of prostate stem cells. Additionally, the approach gives us an optional choice for studying the role of the microenvironment of the maintenance and differentiation of prostate stem cells [41].

Androgen receptor and signaling pathway

Androgen receptor (AR) is known as NR3C4 (nuclear receptor subfamily 3, group C, member 4). It act as a ligand-dependent nuclear transcription factor [45]. As for its structure, it is comprised of three main functional domains, the N-terminal transcriptional regulation domain, the DNA binding domain (DBD) and the ligand-binding domain [46]. It has been well documented that AR has significant effect on the growth, function, and homeostasis of the prostate, and maintain the male sexual phenotype and the development of prostate cancer [47, 48].

Primarily, AR is located in the cytoplasm binding with heat shock proteins (HSPs) and cytoskeletal proteins[49]. HSPs tether AR in the cytoplasm through cytoskeletal proteins like Filamin, and prepare AR for efficient ligand binding by modulating AR conformation [50]. AR could be activated by steroid hormones, typically intracellular dihydrotestosterone and testosterone. After androgen binding to the AR, the changes of the conformation of AR lead to a release of the HSP. The conformation change of AR happens in helices 3, 4, and 12 within the ligand-binding domain (LBD) leading to the formation of activation function 2 (AF2) binding

surface [51]. Also, helix 12 is an amphipathic structure, which is positioned across the ligand-binding pocket, and can reduce ligand dissociation. After released by HSPs, AR interacts with co-regulators such as Filamin-A, ARA70 and importin- α . These proteins bind to the AR nuclear localization signal (NLS) and facilitate the nuclear transporting and dimerization of AR [52]. HSP will be translocated into the cell nucleus and consequently promote the corresponding DNA transcription. AR binds with androgen response elements which are located at the upstream of the target genes. Non-ligand-bound AR will shuttle back to the cytoplasm and re-cycle in prepare for further ligand binding[53]. Abnormal activation of AR in prostate cells has also been demonstrated with intensive correlation with carcinogenesis of the prostate [54].

AR in prostate development and homeostasis

The prostate is an organ which androgen targets for, and the function of androgen to the development and homeostasis has been well established [55]. In the fetal stage, stimulated by androgen, the AR could mediate the development of UGM to a prostate. While in adult prostate, the androgen acts with androgen receptor on stromal cells leading to the secretion of growth factors. The peptide growth factors further regulate epithelial cells and their proliferation, differentiation and apoptosis for the homeostasis maintenance [56].

The inhibition of stromal AR suppresses the proliferation of malignant prostate epithelial cells indicating that the ablation of stromal AR might be a therapeutic strategy for patients with prostate disease [57]. Theoretically, stromal AR is widely accepted as an important factor to modulate the development of prostate due to its various effects. First, the stromal cells and their generated extracellular matrix constitute a microenvironment that regulates the growth of the adjacent epithelial cells. Second, several investigators have demonstrated that during the fetal UGM

development or adult prostatic development, the mesenchymal (stromal) cells express high levels of AR, whereas AR cannot be detected initially in the epithelium. Third, although epithelial AR appears in the later phases of prostate development, it has been confirmed that androgens cannot act directly on the epithelium via epithelial AR to elicit differentiated function. Because of the structure of prostate, stromal cells are the major target of the stimulators in the circulation. As a result, stromal cells serve as paracrine mediators to further secrete biological factors such as epithelial growth factors and fibroblast growth factors [57].

The objectives of the study

In this study, we would like to study 1) how stromal cells regulate the stem cell activity by sphere formation assay. This study will provide the experimental approach of understanding the paracrine factors in regulation of prostate stem cells. Next, we would like to study how AR in the stromal compartment regulates the epithelial stem cell activity and how AR signaling plays a role in this process. We wish to establish an *in vitro* model for further studying the functional roles of AR-regulated downstream factors on prostate stem cell activity.

CHAPTER 2

MATERIALS AND METHODS

Cell lines and cell culture

The mouse embryonic fibroblast cell line NIH 3T3 cells were originally obtained from American Type Culture Collection (ATCC). The NIH 3T3 cell line was transduced by lentiviral vector containing AR gene to create a stable cell line of NIH 3T3+AR. The cell line was grown in the ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) (ATCC 30-2002) with 10% Fetal Bovine Serum (FBS) (Seradigm 3100-500) and 1% Penicillin-Streptomycin (Thermo Fischer Scientific 15140-122). To detect AR transcriptional activity, NIH 3T3 cells and NIH 3T3+AR cells were grown in a 10cm-dish with DMEM with 10% Charcoal/Dextran Stripped Fetal Bovine Serum (JRScientific 43602-500), and treated with 10nM R1881 or DMSO respectively for 24 hours after 24h starving treatment.

Western blot for detection of AR expression

The cell culture medium was removed and the cells were washed with 4°C PBS. Cells were lysed in lysis buffer [radioimmunoprecipitation assay buffer (RIPA buffer)] [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 0.1% SDS, and EDTA-free protease inhibitor cocktail set V (Millipore 539137)]. The adherent cells were collected by scraping the cells from the cell culture dish using plastic cell scraper, and the cell suspensions were subsequently removed into a precooled microcentrifuge tube. After 10 sec sonication by

sonicator (Fisher Scientific), the samples are centrifuged at 4 °C for 15 mins and protein concentration was determined using DC Protein Assay (Bio-Rad).

The protein lysates were mixed with loading buffer to the protein concentration of $2\mu\text{g}/\mu\text{l}$, and then boiled at 95°C for 10 mins. The sample in $20\mu\text{l}$ were loaded into each well of the SDS-PAGE gel for protein separation and subsequently transferred to the nitrocellulose membranes (Bio-Rad). The membranes were blocked in 1X TBS containing 1% Tween-20 (TBST) and 5% milk powder (Lab1 Scientific) for 1 h on a rocker at room temperature, then incubated with the primary antibody solutions against the target protein overnight on a rocker in 4°C cold room. After rinsing membranes 3 times with 10 min each with TBST, the membranes were incubated with the appropriate secondary antibody solutions for 1 hr at room temperature. After washing with 5% milk-containing TBST, all the membranes were imaged after treating with the enhanced chemiluminescence (ECL) Western Blotting detection reagents (Amersham RPN2209).

The use of antibodies were followed with the instructions of manufactory. The AR (441) antibody (Cat# Sc-7305) (1:1000) was purchased from Santa Cruz Biotechnology. GAPDH (14C10) Rabbit mAb (Cat# 2118L) (1:2500), GFP (D5.1) XP(R) Rabbit mAb (Cat# 2956S) (1:5000), Cre Recombinase (D3U7F) Rabbit mAb (Cat# 12830S)(1:1000), Anti-mouse IgG HRP-linked antibody (Cat# 7076S) (1:2500) and Anti-rabbit IgG HRP-linked antibody (Cat# 7074S) (1:5000) were purchased from Cell Signaling.

Quantitative RT-PCR analysis

The total RNA was isolated by the TRIzol™ reagent (Ambion 15596018). Upon addition of 0.3-0.4ml of TRIzol™ reagent per 10^5 - 10^7 cells in the cell culture plate, the lysates were transferred into 1.5ml Eppendorf tube. Then 0.2ml of chloroform (Sigma-Aldrich C2432-1L) per 1ml

TRIzol™ reagent were added for lysis. The sample tubes were shaken vigorously and then centrifuged at 12,000 ×g for 15mins at 4°C. The separation could be observed with a lower red phenol-chloroform phase, an interface and a colorless, upper aqueous phase. The aqueous phase was transferred carefully into a new tube and 0.5 ml of isopropanol (Fisher Scientific A416-4) to the aqueous phase per 1ml TRIzol™ reagent used for RNA precipitation. After centrifugation at 12,000 ×g for 10mins at 4°C, 1ml 75% ice-cold ethanol (Fisher Scientific BP2818-500) per 1ml TRIzol™ reagent was added to each sample for cell lysis and washing the RNA pellet. The tubes were centrifuged at 7,500 - 10,000 ×g for 5mins at 4°C. After air drying the RNA pellet, the total RNA were solubilized in the autoclaved water for downstream analysis.

cDNA were synthesized from total RNA by using the high capacity cDNA reverse transcription kits (Applied Biosystem 4368814) and based on instruction provided. Reverse Transcription Master Mix(2×, 10ul) was added with total RNA and used for reverse transcription reactions. The thermal cycle steps included 25°C for 10min, 37°C for 120min, 85°C for 5min and ended at 4°C. After obtaining the cDNA, the quantitative RT-PCR was implemented in a StepOnePlus™ Real-Time PCR System (Applied biosystems 4376600), in which cDNA, 10μM of forward and reverse primer mix, and SYBR Green FastMix (Quantabio 95072-012) were mixed and nuclease-free water was added to make a total volume of 12.5μl. The primer used are listed in table 1. Each sample is triplicated and the results are analyzed by StepOne Software v2.3(Applied Biosystem). The qPCR reactions are quantified and the expression levels of a target gene are standardized by GAPDH levels in the corresponding samples.

Sphere assay

Prostate tissue was isolated from male C57BL6 mice, and minced into small pieces. The trunks of tissues were further digested in prewarmed DMEM with 10% FBS with 1ml collagenase (190units/ml) and 2mg DNase I (Sigma Aldrich 10104159001) per two tissues at 37°C on a rotator for 90 min. After digestion, the suspension was digested by 0.05% trypsin/EDTA (Gibco 25300-062), and passed through the 22G (Covidien 8881250206) and 27 ¹/₂G (Covidien 8881250362) needles with 10ml syringe (Luer-Lok Tip 306904) for several time to isolate the single cells. The prostate primary cells were isolated after filtering the suspension through a 40µm cell strainer (Fisherbrand 22363547) to remove any cell clumps. Cell numbers were determined by using hemocytometer.

The prostate primary cells were re-suspended in the 50ul PrEGM , and mixed with Matrigel at the volume ratio of 1:1. In the case of the co-culture experiment, the prostate primary cells were mixed with a certain amount of NIH3T3-control or NIH3T3+AR cells, then mixed with the same volume of Matrigel. 100µl of the mixture was plated along the rim of a well of a 12-well plate quickly and then incubated in the 37°C incubator for 45 min. After Matrigel was solidified, 1ml of pre-warmed PrEGM (Lonza CC-3166) supplied with various growth factors was added to the center of each well. The plates were incubated in a 37°C/5% CO₂ incubator. The medium was changed every 5 days. After 10 days incubation, the numbers of the sphere in each well were counted by over 20µ in size meanwhile the sizes were measured under the microscopy.

The primary spheres were used for the passage. The prostate spheres were harvested by incubation with 1ml of medium containing Dispase (20mg/ml) for 1hr at 37°C, and then the suspension was passed through 40 µm cell strainer to remove single cells in the co-culture medium. The prostate spheres were digested by 0.05% trypsin/EDTA and passed through the 22G needed 5 times

followed by a 27 ¹/₂G needle 5 times to break the spheres into single epithelial cells. The obtained single cells were re-plated to form the secondary spheres according to the same method used above.

CHAPTER 3

RESULTS

The prostate sphere formation was enhanced by fibroblast cells in co-culture system.

The Matrigel based 3D-culture model has been widely used for studying the in-vitro maintenance and characterization of prostate stem cell [35]. However, the stromal microenvironment is essential to maintain the niche of stem cells. The contribution of stromal cells to support stem cell activity has been considered in this system. To investigate the maintenance and characteristics of prostate stem cells (PrSCs) under the influence of stromal cells, we have chosen NIH3T3 fibroblasts to study their influence on sphere formation using a co-culture system in vitro where prostate primary cells were cultured with 3T3 cells in 3D Matrigel matrix. Prostate sphere formation was used to represent the PrSCs self-renewal capacity.

As shown in Fig.1, the number of spheres in the co-culture group was 175 as a mean which is significantly higher than 4 as a mean in the control group where the prostate primary cells were cultured alone. We further dissociated the spheres from the co-culture group into single cells to study the self-renewal potential of prostate epithelial cells. The dissociated cells from primary spheres were passaged and cultured with or without NIH 3T3 cells. Similarly, the number of spheres in the co-culture group is higher in comparison to that without NIH 3T3 cell group (Fig.2). The results show that the co-culture of prostate primary cells with NIH 3T3 cells promotes prostate sphere formation, suggesting that the self-renewal capacity of prostate epithelial cells was enhanced in the presence of NIH 3T3 cells.

The prostate sphere formation was further enhanced by fibroblast cells overexpressing AR.

It has been widely confirmed that AR levels in prostate epithelial cells have significant effect on the growth, function, and homeostasis of the prostate [45]. However, the functional role of stromal AR is not well established. AR plays a critical role in prostate development through stage-specific regulation of androgenic effects on epithelial cells. Much of this activity is believed to be mediated by the release of paracrine factors, which are independent of the presence of epithelial AR [58]. Stromal cells in human prostate include fibroblasts and smooth muscle cells, which contribute to the synthesis of the extracellular matrix. Together, the stromal cells and extracellular matrix generate a microenvironment that regulates the growth of the adjacent epithelial cells [57]. Hence, to take into consideration of the AR function in the stromal cells, we studied the functional role of AR in vitro using the above established co-culture system, and the effect of AR over-expression in the NIH 3T3 cells on the sphere formation was examined.

Bi-cistronic lentiviral vector carrying AR gene was used in the study. The AR expression is under the control of the ubiquitin promoter. The vectors containing GFP reporter gene driven by CMV promoter were used as a control (Fig. 3a). NIH 3T3 cells were transduced with AR or control lentiviral vector, and were sorted based on the fluorescence intensity of GFP (Fig. 3b). The GFP positive cells were further expanded. As shown in Figure 3c, all the infected cells were GFP positive (Fig.3c). We confirmed the AR expression via RT-qPCR. The AR expression in AR-vector transduced NIH 3T3 cells is 683 times higher than that of NIH 3T3 cells transduced with control vector (Fig.3d). We further confirmed the over-expression of AR by Western blot (Fig. 3e). While expression levels of AR was not detected in the NIH 3T3 cells or 3T3 cells expressing the control vector (Fig. 3e), the sorted NIH 3T3 cells expressing AR showed similar expression

levels of AR to the C4-2B cells (positive control). The established 3T3 cells expressing control and AR were used to study the effect of AR expression in NIH 3T3 cells on the sphere formation of prostate primary cells.

As expected, the number of sphere in the co-culture groups was higher in comparison with that of primary epithelial cell alone. More importantly, the number of the spheres in the NIH 3T3-AR group was significantly higher than that in the 3T3-control group (Fig. 4a). However, the size of the spheres had no significant difference between two groups where prostate primary cells co-cultured with NIH3T3 cell expressing AR or control. In fact the size in the groups co-cultured with NIH 3T3 cells was slightly smaller than that in the prostate primary cells alone (Fig. 4b).

The effect of AR activation on prostate sphere formation

It has been accepted that the presence of androgen could activate the AR in various cell lines derived from prostate by affecting AR phosphorylation, nuclear transport and transcriptional activity. [59] R1881, also known as methyltrienolone, is a synthetic anabolic-androgen steroid (AAS) and its effects on the AR by increase AR stability and nuclear translocation of AR have been revealed in many reports. [60] Accordingly, R1881 was used to get insight into the role of AR in our studied co-culturing systems. In this experiment, the prostate primary cells were cultured alone, co-cultured with either NIH 3T3 +control cells, or NIH 3T3+AR cells, and with or without the R1881 treatment.

As shown in the Figure 5, the number of spheres in the co-culture groups was higher compared with the prostate primary cells alone. Additionally, the sphere number in the group of 3T3+AR co-cultured cells was higher than that in the 3T3+control group. However, the sphere number was significantly lower in the 3T3+control+R1881 or 3T3+AR+R1881 groups in comparison to that

of 3T3+control+vehicle or 3T3+AR+vehicle, respectively. In addition, the reduction of the sphere number between the NIH 3T3+ AR with/without R1881 groups was 2.3 folds in comparison with that between the NIH 3T3+control with/without R1881 groups (Fig. 5a).

Similar to the data in Figure 4, the size of spheres had no significant difference between the groups where prostate primary cells co-cultured with NIH 3T3 cell expressing AR or control. However, the size of spheres in the groups co-cultured with NIH 3T3 cells expressing AR or control with/without R1881 treatment was smaller than that in the prostate primary cells alone. (Fig. 5b).

CHAPTER 4

DISCUSSION

The importance of the epithelial stem cells has been noted in clinic because of their potential use for organ replenishment [61] as well as their capacity for initiation a cancer [62]. Because of the self-renewal and differentiation capacity of epithelial stem cells, they are able to replace lost or damaged tissue to ensure proper organ function during the normal life span of an adult organ [63]. Also, epithelial stem cells are suspected to be the cell-of-origin of many cancers due to their inherent self-renewal capacity and long lived nature [64]. Therefore, the investigations of the properties and basic biological processes of the epithelial stem cells and the importance of their surrounding environment will be useful for developing new therapeutic methods.

The prostate is an ideal model for studying the properties of epithelial stem cells. Based on the androgen-respond regression and regeneration experiment of prostate gland, the prostate stem cell was discovered [28], offering a possibility for organ regeneration and the treatment of prostate disease although the prostate is not vital for human survival. The pathology of benign prostatic hyperplasia (BPH) are believed to be the expansion of prostate stem cells in many studies [65]. More important, the prostate stem cells are androgen-independent, which share the same property with androgen-independent (castration-resistant) prostate cancer (CRPC) and suspect to have the relationship with CRPC [66]. Herein, identifying the properties of prostate stem cells may provide new therapeutic target for prostate diseases.

Compared with two-dimensional cell culture system where primary cells quickly lose their self-renewal potential [40], a three dimensional culture system was developed exhibiting satisfied

performance in maintaining the self-renewal and differentiation properties of the prostate primary cells in-vitro [41]. In this system, the prostate primary cells were suspended into the semi-solid matrix containing PrEGM and Matrigel. PrEGM is the serum free medium containing basal medium and supplements such as growth factors including EGF, FGF and cytokines for maintenance and expansion of prostate epithelial cells. Matrigel comprised of extracellular matrix (ECM), and together with PrEGM closely mimics the basement membrane which is ECM-rich and embraces the basal cells in the prostate gland in an in-vitro environment[67]. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma which is rich in ECM proteins such as laminin, collagen IV, heparan sulfate proteoglycans, entactin/nidogen, and a number of growth factors. The biological and chemical components of the prostate basal membrane is unknown, and the Matrigel might not fully represent the basal membrane microenvironment which epithelial cells are surround with under the physiological conditions. However, it has been well-demonstrated that Matrigel efficiently maintain the stem cells and form sphere. The sphere has been shown to contain basal and luminal cells, and can be passaged many generation in the Matrigel [35].

In our study, the sphere-forming assay was used to study the self-renewal and sphere forming capacity of prostate primary cells. PrSCs are able to form clonal spheres when cultured in the three-dimension system. The spheres are comprised of hundreds of cells with cells possessing higher self-renewal capacity in the outer layers while cells toward the center display a more mature and differentiated phenotype which is similar to the structure of the native prostate gland [41]. Self- renewal capacity can be studied by using this sphere assay when the spheres are dissociated and re-plated to form a new generation of spheres [41]. In contrast, an in-vivo prostate regeneration assay is a gold standard to measure prostate stem cells capability. In the assay, the PrSCs are

combined with stromal cells and implanted under the kidney capsule or subcutaneously to the immunodeficient mice. A prostate stem cell will maintain the ability to regenerate prostatic tubules. The regenerated tubules are as same as the primary prostate tubules, with an outer basal layer, an inner luminal layer and rare neuroendocrine cells [68]. However, compared with the in vivo prostate-regeneration assay which takes 6-10 weeks, the experiment period of in vitro sphere-forming assay is relative short which last only 10 days. Hence, we choose the sphere-forming assay as the method to evaluate the activities of prostate primary cells suggesting the activity of PrSCs. Our study demonstrated that the co-culture of prostate primary cells with NIH 3T3 fibroblast cells promotes prostate sphere formation, suggesting that the self-renewal capacity of prostate stem cells was enhanced in the presence of NIH 3T3 cells. The function of prostate stromal cells to the development and maintenance of prostate has been well established [55] [69]. The prostate stromal cells consist of fibroblasts and smooth muscle cells which contribute to the synthesis of ECM. The ECM and the stromal cells contribute to the microenvironment of the development of epithelial cells. In the previous study, co-culture of primary prostate cells with the urogenital mesenchymal cells (UGSM) was performed for sphere formation. The UGSM is embryonic stromal cell, and the results suggested that UGSM promotes sphere formation of prostate primary cells [70]. The focus of the current study is to examine the contribution of fibroblast cells, using NIH 3T3 cells as a model, to sphere formation. The sphere number in the co-culture group is significantly higher than that of the culture of prostate primary cells alone suggesting that NIH 3T3 cells can enhance the sphere forming capacity of prostate primary cells. When the primary spheres form co-culture group were dissociated and passage into new generation, the number of sphere forming from the co-culture group was still higher, suggesting that induction of NIH 3T3 cells can enhance the self-renewal capacity of the prostate epithelial cells. Figure 2b shows the sphere number derived from

dissociated cells from primary spheres. The ratio forming a sphere from prostate primary cells is different to that from dissociated cells primary spheres. That is the reason that less sphere was formed in Figure 2b. The co-culture system is similar to primary cells growing with the feeder cells. However, feeder cells consist of a layer of cells unable to divide, and provide extracellular secretion to help other cells to proliferate[71]. Therefore, feeder cells differ from the co-culture system because in the co-culture, 3T3 cells are not treated chemically or physically to inhibit its division. These cells are alive and able to proliferate. But similarly, these cells likely secrete growth factor and other biological factors to support the sphere formation. Other types of cell lines may have the same function as 3T3 cells if the secreted factors are similar. However, this will need to be tested. Stromal-epithelial interaction is essential for maintaining the niche of stem cells and regulating organ development and cancer progression. Several studies have determined that stromal-derived factors, including ligands in the Wnt, FGF, and TGF- β family, contribute to prostate epithelial homeostasis, regeneration, and transformation [72] [73] [74]. In our study the NIH 3T3 cells, as an embryonic fibroblast, acted as the stromal compartment inducing the sphere formation probably because of the secreted factors or other biological factors such as ligands in the Wnt, FGF, and TGF- β family. It will be interesting to study which biological factors contribute to the elevation of sphere formation. Biochemistry such as Mass spectrometry and molecular biology including gene knock down approaches will be required to explore these factors.

Our study demonstrated that the prostate sphere formation was further enhanced by fibroblast cells overexpressing AR. The critical role of UGSM AR on the overall development of fetal prostate has been established [75]. Yeh's study has shown that both luminal infolding and the level of IGF - 1 production in mouse prostate were reduced when AR gene was knocked out in prostate smooth muscle cells. Also, Yeh and coworker generated tissue-selective knock-out mice where

AR gene is deleted in stromal fibroblasts. They found that in the prostate of these mice, the epithelial proliferation was decrease while the apoptosis was increase. Further mechanistic studies illustrated the stromal fibroblast AR knock-out mice had defects in the expression of prostate stromal growth factors [76]. However, how the AR in stromal fibroblast affect the activity of PrSCs is still unclear. Since the AR expression of NIH 3T3 fibroblast cannot be detected by the immunoblot method in our study, we decided to overexpress the AR in the NIH 3T3 cells by lentiviral vector to study its functional role on the activity of PrSCs in-vitro. Our study illustrated that the sphere forming capacity of PrSCs was increased when co-cultured with stromal fibroblasts overexpressing AR. The result suggests that AR in NIH 3T3 fibroblast cells can elevate the sphere-forming capacity of PrSCs, but how AR in NIH 3T3 cells affect the sphere-forming capacity of PrSCs mechanically is still unclear. Further experiments to analyze AR regulated down-stream genes should be performed. This analysis includes RNA-seq and/or Mass spectrometry to understand AR-mediated growth factors or other signaling pathways to regulate the stem cell activity and the sphere formation.

To further investigate the functional role of AR in NIH 3T3 cells to the activity of prostate primary cells, the androgen R1881 was used in the co-culture system. In Jon A. Kempainen's study, they used transfected COS cells to study the activity of the recombinant AR in the presence of various steroids [77]. It was found that under low steroid concentration, AR had high binding affinity with biologically active androgen; while at high steroid concentration, its binding affinity with AR would be reduced, and the nuclear transport and transcriptional activity of AR was also reduced [77]. When the experiment group was treated with 10nM R1881, the sphere number was significantly reduced in the 3T3+control+R1881 or 3T3+AR+R1881 groups in comparison with 3T3+control+vehicle or 3T3+AR+vehicle, respectively. The results suggested that 10nM of

R1881 might be high enough to reduce the nuclear transport and transcriptional activity of AR in stromal cells. It is highly possible that within a range of AR activity, AR can enhance the sphere forming potential of prostate primary cells, but whenever the AR activity exceeds the range, the sphere forming activity is reduced with the increase of AR activity. Although luminal cells express high level of AR and basal cells have low AR expression, there are AR expression in the luminal, basal and stromal cells in the native prostate gland. Stromal AR is believed to be essential for prostate developmental growth and morphogenesis through paracrine signals. R1881 is an AR agonist, which specifically activate AR signaling. Based on our results, in the groups of primary cells alone, there's no significant difference of the sphere number with or without R1881 suggesting that R1881 don't have significant effect on the primary cells. This is evident in the primary cells alone group treated with/without R1881, which there is no significant difference in the sphere number. Therefore 10nM of R1881 cannot be toxic to prostate primary cells. In the groups of co-culture. Additionally, there's no evidence that the growth and proliferation of NIH 3T3 cells were reduced or the apoptosis was enhanced when treating with R1881, further suggesting that 10nM of R1881 should not have cytotoxic effect on NIH 3T3 cells. To further confirm that 10nM of R1881 is not toxicity on NIH 3T3 cells, the viability of NIH 3T3 with/without overexpressing AR in response to a dose range of R1881 should be performed. In order to study the contribution of AR signaling, different concentration of R1881 should also be used to treat in the co-culture to study if the different AR activity changed sphere formation. Additionally, expression levels of AR downstream genes should be measured. RT-qPCR will be performed to detect the downstream genes of AR of interest after adding R1881 or RNA-seq can be used to study the changes of expression level of the downstream genes.

CHAPTER 5

CONCLUSIONS

Taken together, NIH 3T3 fibroblasts cells increase the prostate sphere formation of prostate primary cells, and enhance the secondary sphere formation from dissociated cells from primary spheres. While co-culture with NIH 3T3 fibroblast alone increase 20 folds in sphere formation, over-expression of AR in NIH 3T3 cells further enhance 20% of the sphere formation, suggesting that biological factors secreted by 3T3 cells likely contribute significantly to sphere formation. However, activation of AR signaling by 10 nM R1881 inhibited the sphere forming potential. It is possible that the secretion of these biological factors such as ligands in the Wnt, FGF, and TGF- β family might have been interfered by the AR activation. Therefore, the stimulation of the sphere forming requires optimal levels of AR activity. Our future study should investigate which molecular factors mediate AR signaling to affect the sphere forming potential, which provide a reference in regulation of prostate stem cells.

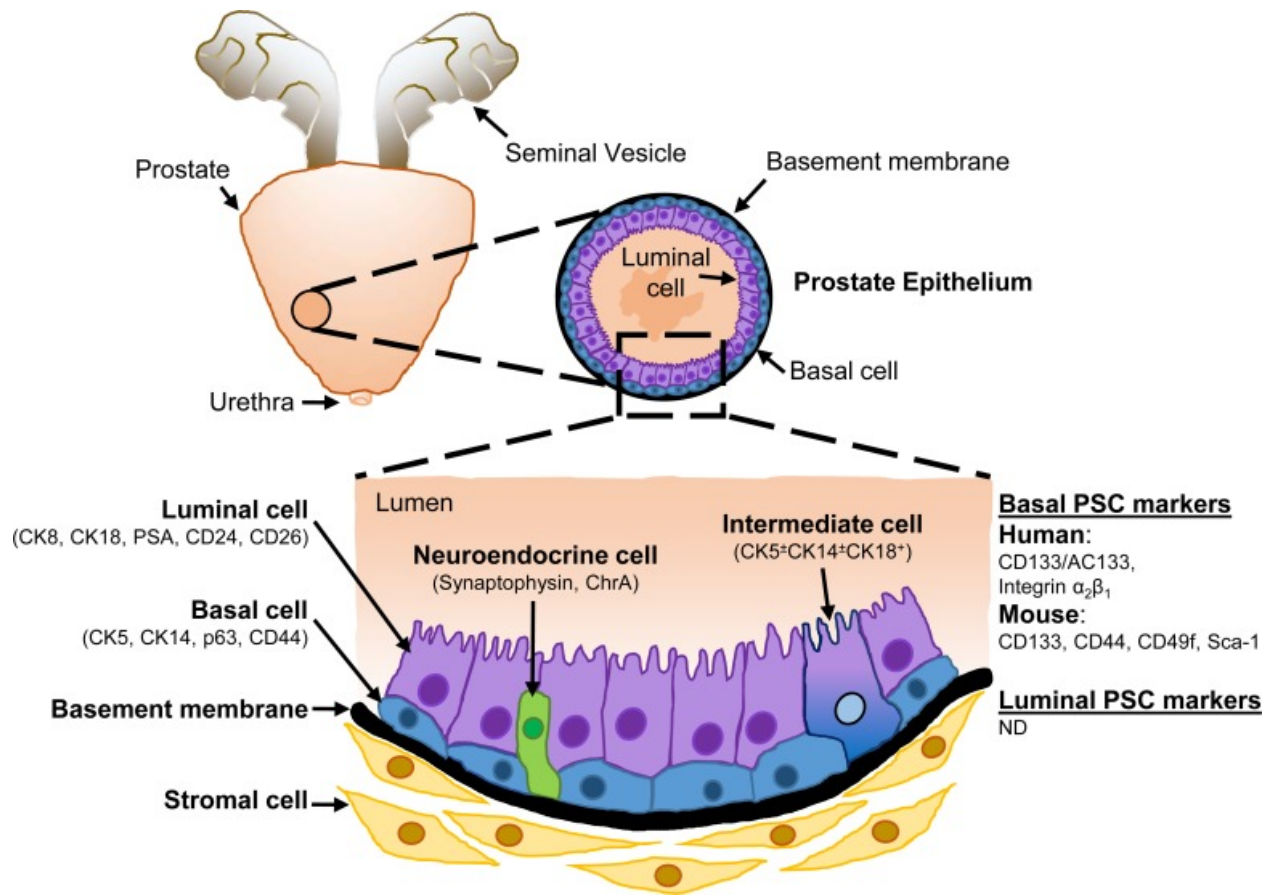


Figure 1. The structure features of prostate [11]. The prostate has two major cell types: epithelial and stromal. Within the prostate epithelium, it consists of an inner layer of secretory luminal cells. Basal cells form a continuous layer of cells around the luminal cells and in contact with the basement membrane, which serves as a barrier between the epithelium and stromal compartment. There are also intermediate cells and rare neuroendocrine cells. Different cell types are identified by different cell surface markers. The figure is adapted from Rybak et al. (2015)

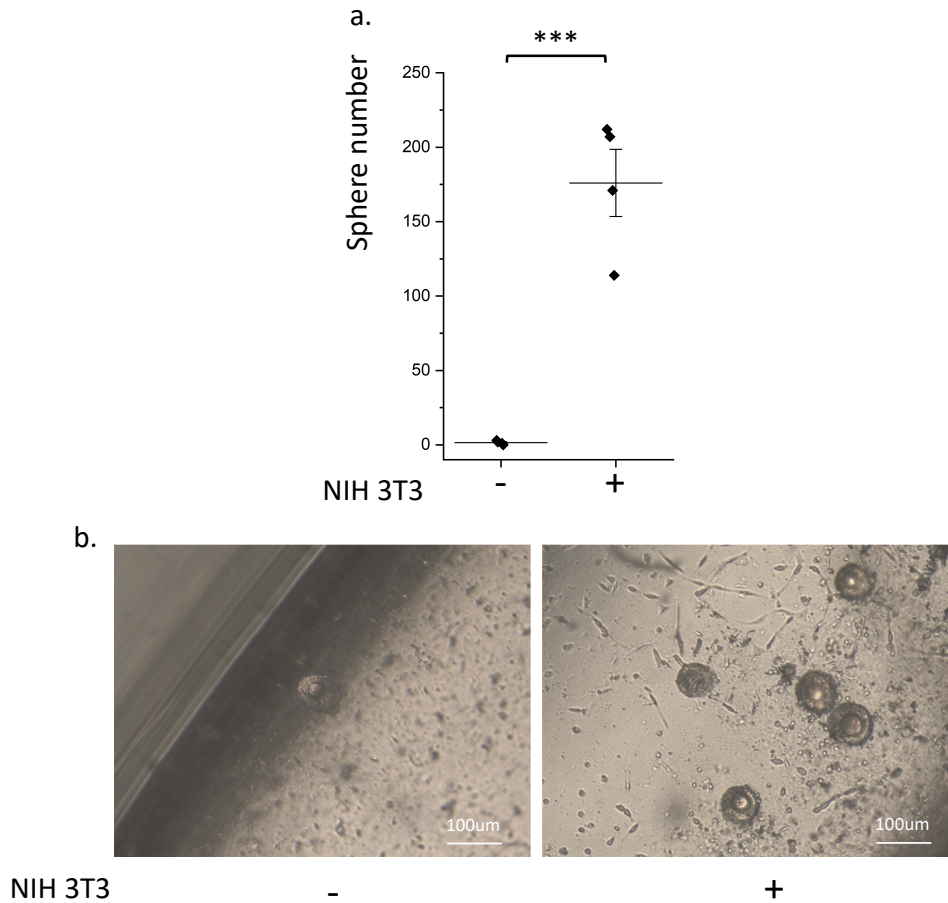


Figure 2. The effect of NIH 3T3 fibroblast cells on the formation of prostate primary sphere.

The prostate tissue is dissected from male C57BL6 mice, and digested into single cells by incubating with collagenase and DNase I and passing through the needles. (a) After the prostate primary cells were isolated, 35,000 prostate primary cells were grown in the Matrigel alone, or co-cultured with 5,000 NIH 3T3 cells. After 10 days culturing, the number of the sphere in each well were counted under microcopy. The data represent the mean $\pm$ SEM. *** means $p \leq 0.001$. (b) A representative image was taken under the microcopy with $100\times$ magnification supported by the ZEN software.

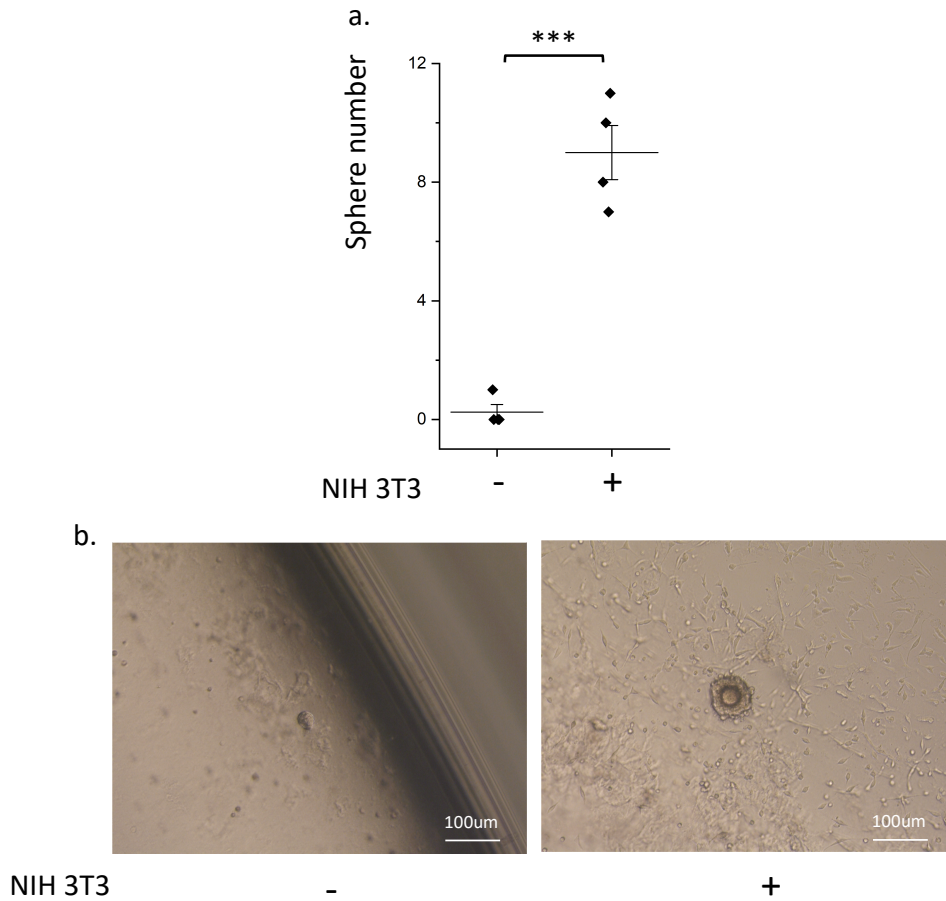
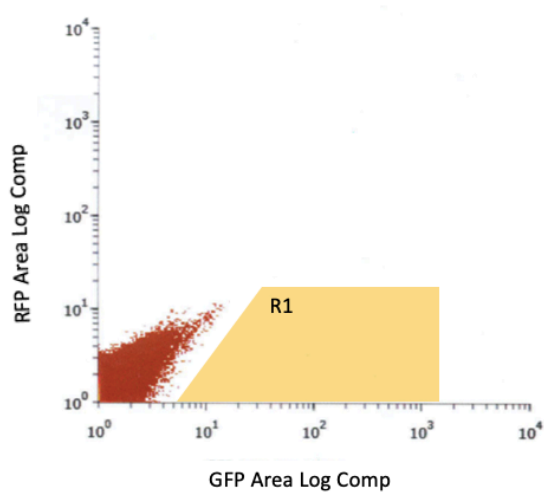


Figure 3. The effect of NIH 3T3 fibroblast cells on the passage of primary spheres. The spheres co-cultured with NIH 3T3 cells were harvested and dissociated into single cells. (a) After dissociating the sphere into single cells, 5,000 PrECs were grown in the Matrigel alone, or co-cultured with 5,000 NIH 3T3 cells. After 10 days, the number of the sphere in each well were counted under microcopy. Each group had four replicates. The data represent the mean $\pm$ SEM. *** means $p \leq 0.001$. (b) A representative image was taken by the software of ZEN.

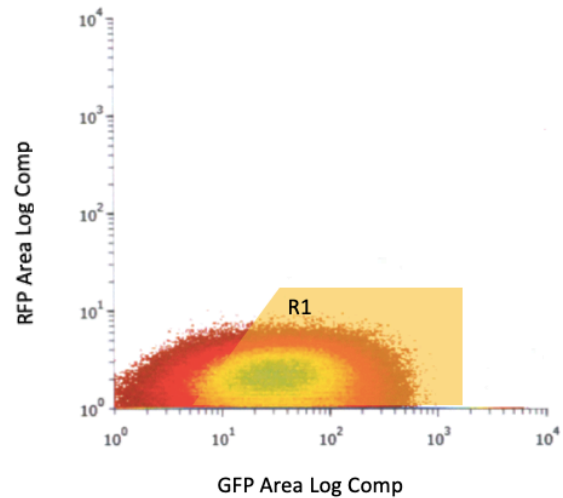
a.



b.

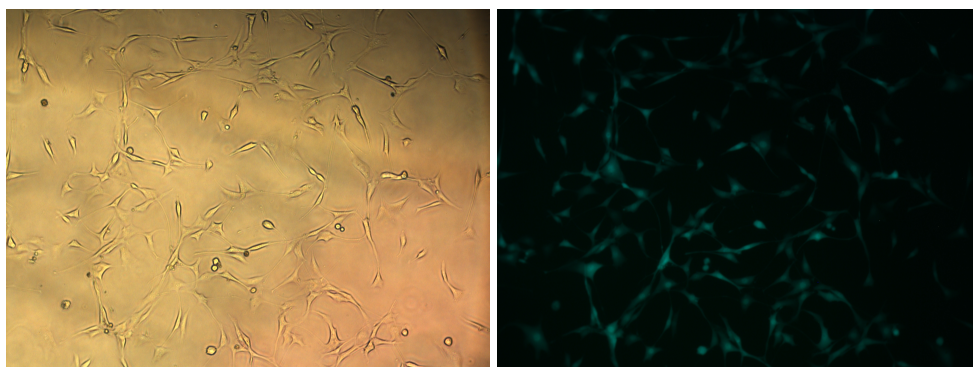


NIH 3T3

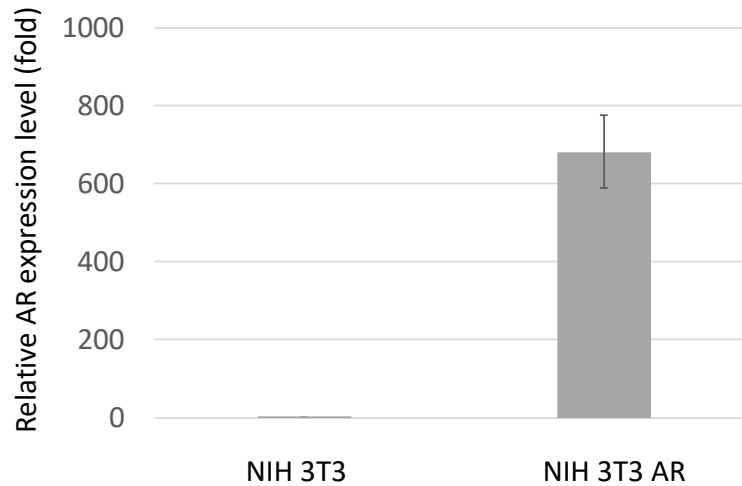


NIH 3T3 AR

c.



d.



e.

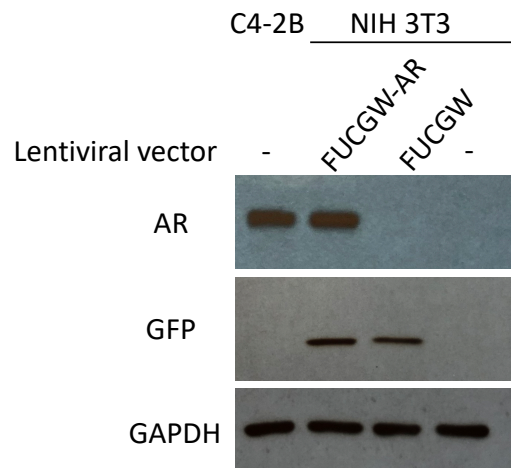


Figure 4. Overexpression of AR in NIH3T3 cells. (a) The scheme of lentiviral vector of FUCGW-AR. AR gene was expressed in a bi-cistronic lentiviral vector, and is regulated by the ubiquitin promoter. Expression of GFP gene is regulated by the CMV promoter. (b) NIH3T3 cells were transduced with AR (FUCGW-AR) or control vector (FUCGW) by lentiviral infection, and the cells were sorted with green fluorescence by Bio-Rad S3 cell sorter. Compared

with the sorting histogram of NIH 3T3 cells (GFP negative), the cells in the gate R1 were the population of GFP positive and were selected. (c) All the cells were expression GFP and plated into the 10 cm culturing dish. (d) The cells were lysed and expression levels of AR and GAPDH were measured via qPCR. AR expression level was normalized to GAPDH and expression levels in NIH 3T3 cells expressing control vectors was normalized as 1. (e) The expression levels of AR, GFP, GAPDH were examined in the C4-2 cells (Control), 3T3 cells, and 3T3 cells expressing control vector or AR by immunoblotting. GAPDH was used as the loading control.

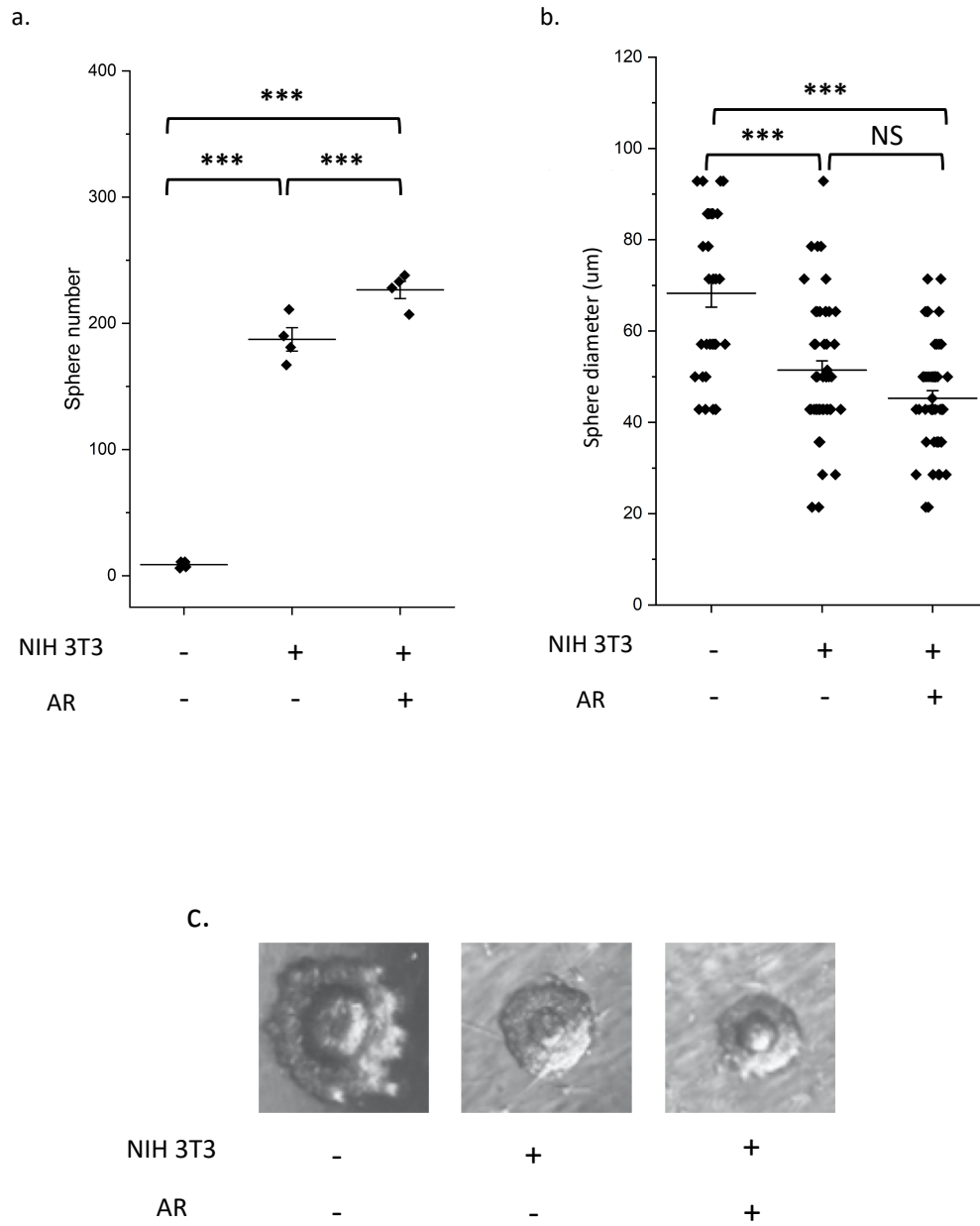
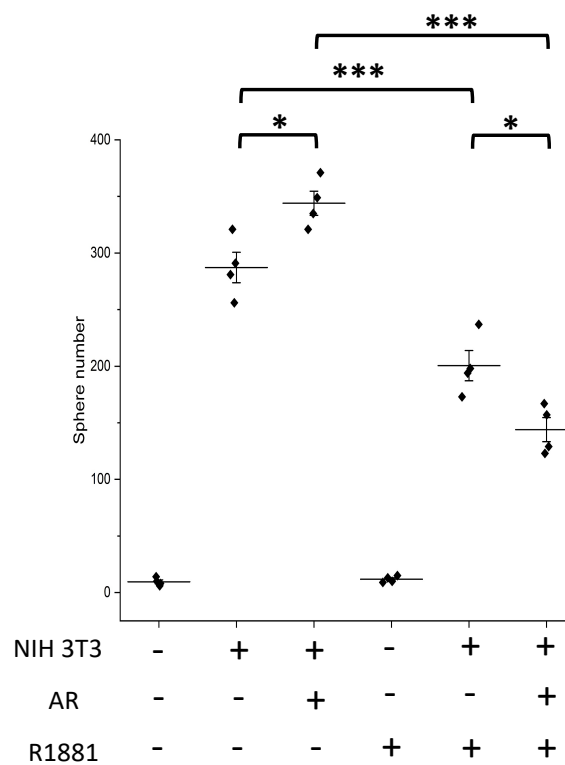


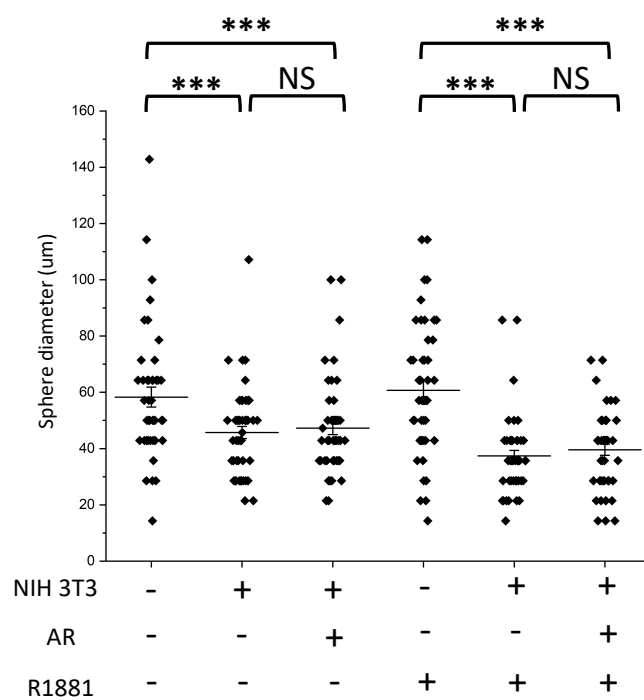
Figure 5. The effect of over-expression of AR in NIH 3T3 cells on the production of primary sphere. Prostate tissues were isolated from the C57Bl6 mice. The prostate tissue were dissociated into single cells. (a) The isolated prostate primary cells were co-cultured with or without NIH 3T3 cells expressing control vector or AR for the culturing period of 10 days. 10,000 prostate primary

cells were grown in the Matrigel alone, or co-cultured with 1,000 NIH 3T3 cells or NIH 3T3 AR cells. The number of the sphere in each group were counted under microcopy. (b) Representative images were taken from each group. The size of spheres in each image were measured and subjected to the analysis. Each group had four replicates. The data represent the mean $\pm$ SEM. *** means $p \leq 0.001$, NS means $p > 0.05$. (c) Representative images of the sphere were taken from each group.

a.



b.



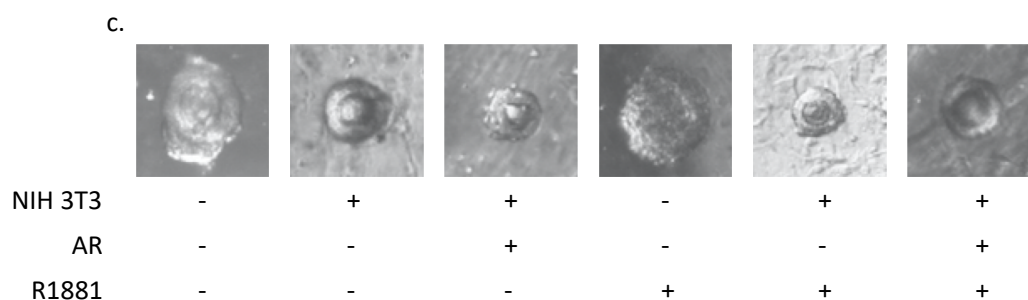


Figure 6. The effect of androgen-AR signaling on the prostate sphere formation. Prostate primary cells were isolated from prostate tissues of C57Bl6 mice. (a) The isolated prostate cells were co-cultured with or without NIH 3T3 expressing control vector or AR cells, and treated with/without 10 nM R1881. 10,000 prostate primary cells were grown in the Matrigel alone, or co-cultured with 1,000 NIH 3T3 cells or NIH 3T3 AR cells. R1881 dissolved in DMSO was added directly into the PrEGM at concentration of 10 nM every three days as experiment group, while the control group contained the same amount of blank DMSO. After 10 days culturing was completed, the number of the spheres were counted in each treatment group under microcopy. (b) Representative images were taken from each group. The size of spheres in each image were measured and subjected to the analysis. Each treatment group had four replicates. The data represent the mean $\pm$ SEM. *** means $p \leq 0.001$, ** means $p \leq 0.01$, * means $p \leq 0.05$, NS means $p > 0.05$. (c) Representative images of the sphere were taken from each group.

Table 1. Forward and Reverse Primer Sequence for qPCR

GAPDH Forward	5'-AGGTCGGTGTGAACGGATTTG-3'
GAPDH Reverse	5'-TGTAGACCATGTAGTTGAGGTCA-3'
AR Forward	5'-CCTGGCTTCCGCAACTTACAC-3'
AR Reverse	5'-GGACTTGTGCATGCGGTACTCA-3'

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