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A genetic screen in *Drosophila* for regulators of human prostate cancer progression

Saya Ito *, Takashi Ueda, Akihisa Ueno, Hideo Nakagawa, Hidefumi Taniguchi, Naruhiro Kayukawa, Tsuneharu Miki

Department of Urology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kajii-cho, 465, Kyoto City, Kyoto 602-8566, Japan

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ABSTRACT

To uncover the mechanism by which human prostate cancer progresses, we performed a genetic screen for regulators of human prostate cancer progression using the *Drosophila* accessory gland, a functional homolog of the mammalian prostate. Cell growth and migration of secondary cells in the adult male accessory gland were found to be regulated by *paired*, *N-cadherin*, and *E-cadherin*, which are *Drosophila* homologues of regulators of human prostate cancer progression. Using this screening system, we also identified three genes that promoted growth and migration of secondary cells in the accessory gland. The human homologues of these candidate genes – *MRGBP*, *CNPY2*, and *MEP1A* – were found to be expressed in human prostate cancer model cells and to promote replication and invasiveness in these cells. These findings suggest that the development of the *Drosophila* accessory gland and human prostate cancer cell growth and invasion are partly regulated through a common mechanism. The screening system using the *Drosophila* accessory gland can be a useful tool for uncovering the mechanisms of human prostate cancer progression.

1. Introduction

Identification of novel factors regulating cancer cell growth and invasion will lead to better understanding of the mechanisms of cancer progression and the identification of new therapeutic targets. *Drosophila* is often used as a model for the study of human cancer progression [1]. Because the *Drosophila* eye has several advantages for genetic screening, novel regulators of human cancer progression have been identified by analyzing the *Drosophila* eye in gain- or loss-of-function conditions. However, such factors are not tissue-specific, and inhibition of the function of such factors in humans may cause injury in tissues other than the target tissue. Establishment of a screening system using tissues homologous to the target human tissue may be useful for the identification of tissue-specific therapeutic targets.

The *Drosophila* male accessory gland has functions similar to those of the mammalian prostate gland and seminal vesicle [2,3]. The accessory gland secretes several proteins, such as sex peptides, which are transferred to the female and required for physiological and behavioral responses in the female upon mating [4–6]. The accessory gland consists of two types of binucleate cells: main cells and secondary cells [7]. These two cell types in the adult accessory gland are crucial for male fecundity [8,9] and develop in response to mating activity in different ways [7]. Growth occurs preferentially in secondary cells in response to mating activity [3]. In addition, some distally located secondary cells migrate within the gland and are transferred to females upon mating [3]. BMP signaling, which is known to induce cell growth and migration in human cancers [10], promotes cell growth and migration of *Drosophila* secondary cells [3]. Study of the growth and migration of *Drosophila* secondary cells may help to uncover the mechanisms of cancer cell invasion. In this report, we designed a genetic screening system using the secondary cells of the *Drosophila* accessory gland, which is homologous to the human prostate, to uncover the mechanisms of human prostate cancer progression.

2. Materials and methods

2.1. Fly genetics

Esg^{ts}-GAL4, *UAS-GFP$_{nls}$*; *act > CD2 > GAL4*, *UAS-FLP* were provided by Edgar and Wilson [3,11]. RNAi-expressing strains (NIG-Fly) were obtained from the Genetic Strain Research Center, National Institute of Genetics (stock numbers are shown in Table 1).

When specific expression of *UAS-RNAi* in adult secondary cells under *esg^{ts} Flp-Out* control was required, RNAi-expressing males

* Corresponding author. Fax: +81 75 251 5597.
E-mail address: itosaya@koto.kpu-m.ac.jp (S. Ito).

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Table 1
Genetic screening in *Drosophila* male accessory gland for metastatic regulators. Twenty-five genes highly expressed specifically in the accessory gland were selected by reference to ***modENCODE high-throughput RNA-seq data*** [17]. RNAi-expressing strains (NIG-Fly) targeting the twenty-five genes were used for genetic screening. Males containing *UAS-RNAi* genes were crossed with females containing *esg^{ts} Flp-Out* and cultured at 18 °C. Individual, newly eclosed F1 males were crossed to individual virgin *w^{1118}* females and cultured at 28.5 °C. 10 days after eclosion, accessory glands were dissected and observed using a fluorescence microscope. The number of flies and total observed flies are shown in each box. (i) Number of GFP-marked secondary cells altered in RNAi-driven knockdown flies. A Chi-square test was performed between 'decrease' and 'normal'. *$P < 0.05$. (ii) The number of migrated secondary cells was also altered in some knockdown flies. Migrated cells were seen in about 35% of wild-type males. We determined the migration frequency was occurred in 35% of total flies, and a Chi-square test was performed between the group with 'more than or equal to two migrated-cells' and the one with 'less than one migrated cell'. *$P < 0.05$. *wild-type* means offspring of *w^{1118}* males, which were crossed with *esg^{ts} Flp-Out* females.

Symbol	Name	Annotation ID	NIG-Fly Stock ID	(i) cell number		(ii) migration		
				decrease	normal	≧2 cells	1 cells	0 cells
Aph-4	Alkaline phosphatase 4	CG1462	1462R-2	-	1/1	-	-	1/1
AnxB11	Annexin B11	CG9968	9968R-1	2/2	-	-	-	2/2
gbb	glass bottom boat	CG5562	5562R-3	-	6/6	1/6	1/6	4/6
scf	supercoiling factor	CG9148	9148R-2	3/4	1/4	-	2/4	2/4
sel	seele	CG12918	12918R-2	13/19 *	6/19	3/19	-	16/19
βCop	β-coatomer protein	CG6223	6223R-2	1/4	3/4	2/4	1/4	1/4
CG5862		CG5862	5862R-1	3/13	10/13	-	1/13	12/13
CG4164		CG4164	4164R-4	2/13	11/13	2/13	1/13	10/13
wbl	windbeutel	CG7225	7225R-1	-	2/2	-	-	2/2
CG5112		CG5112	5112R-1	2/3	1/3	-	-	3/3
CG4623		CG4623	4623R-3	-	3/3	-	-	3/3
Dp1	Dodeca-satellite-binding protein 1	CG5170	5170R-1	5/5 *	-	-	1/5	4/5
KdelR	KDEL receptor	CG5183	5183R-1	1/3	2/3	-	-	3/3
CG31872		CG31872	31872R-2	-	4/4	-	1/4	3/4
CG17271		CG17271	17271R-1	2/2	-	-	-	2/2
CG11864		CG11864	11864R-2	6/28	22/28	11/28 *	1/28	16/28
Npc2b	Niemann-Pick type C-2b	CG3153	3153R-3	5/10	5/10	2/10	-	8/10
PH4αNE2	prolyl-4-hydroxylase-alpha NE2	CG9720	9720R-2	6/11	5/11	6/11 *	-	5/11
CG1418		CG1418	1418R-1	2/9	7/9	2/9	-	7/9
CG1316		CG1316	1316R-1	-	8/8	3/8	1/8	4/8
Spn28F	Serpin 28F	CG8137	8173R-2	1/3	2/3	-	-	3/3
bai	baiser	CG11785	11785R-2	-	8/8	3/8	3/8	2/8
TRAM	TRAM	CG11642	11642R-3	1/1	-	-	-	1/1
Ostγ	Oligosaccharide transferase γ subunit	CG7830	7830R-1	-	3/3	2/3 *	1/3	-
MrgBP	MrgBP	CG13746	13746R-2	11/11 *	-	1/11	-	10/11
prd	paired	CG6716	6716R-1	11/23 *	12/23	4/23	1/23	18/23
E-cad	shotgun	CG3722	3722R-2	8/12 *	4/12	4/12	2/12	6/12
N-cad	Cadherin-N	CG7100	7100R-3	8/9 *	1/9	1/9	-	8/9
wild-type				-	11/11	-	4/11 (35%)	7/11 (65%)

The number of flies/the number of observed total population.
*$P < 0.05$ [(i) decrease vs. normal; (ii) ≧ 2 cells vs. 0–1 cell (migration frequency is 35%)].

were crossed with *esg^{ts} Flp-Out* females and cultured at 18 °C [3]. Individual newly eclosed F1 males were crossed to individual virgin *w^{1118}* females and cultured at 28.5 °C. Ten days after eclosion, accessory glands were dissected out and observed using a fluorescent microscope (OLYMPUS). For detailed imaging analysis, dissected accessory glands were fixed in PBS with 4% paraformaldehyde and 0.1% Triton X-100 at 25 °C for 20 min and washed with PBS [12]. The glands were then mounted with VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories, CA, USA) and imaged using a Zeiss 510 laser confocal microscope.

2.2. RT-qPCR

Accessory gland tissues were dissected from mated adult males. Total RNA was then isolated using ISOGEN reagent (Wako, Osaka, Japan). Reverse transcription (RT) was performed using PrimeScript RT Master Mix (Takara, Shiga, Japan) according to the manufacturer's instructions. cDNAs were quantified by real-time PCR using SYBR qPCR mix (Toyobo, Osaka, Japan) and a Thermal Cycler TP800 (Takara). RT primers were as follows: *prd* (s) 5′-GACC GTAACCGCCTTTGCT-3′, (as) 5′-GAGATCACACAGGGCCGAAT-3′, *dMrgBP* (s) 5′-AAGCTGAAGGAACTGGACGA-3′, (as) 5′-CATTGGTCT CTGCTGTTGGA-3′, *sel* (s) 5′-CCTACAAGAGCAACGGGAAA-3′, (as) 5′-GTCGCAGTAGCTCGCTTGTT-3′, *CG11864* (s) 5′-GGCTGCAATA CCGTTCACTT-3′, (as) 5′-TTGGGACACATGCTGATGTT-3′, *rp49* (s) 5′-T TGAGAGTTCTTGTAACGTGGTCGG-3′, (as) 5′-AATGACAATTGAACT CGGCACTCGC-3′, h*MRGBP* (s) 5′-CCTCAGGCTCTCAGGTGAAC-3′, (as) 5′-CGTAGCGCTAATCCATGTCA-3′, *hCNPY2* (s) 5′-GACCATGCC CTGCACATATC-3′, (as) 5′-TAAAAGGCATTGCCACCATT-3′, *hMEP1A* (s) 5′-AAAGGCCAAGGAAGTGACCT-3′, (as) 5′-ATGTGGGGCAGAGAG ATGAC-3′, GAPDH (s) 5′-GCACCGTCAAGGCTGAGAAC-3′, (as) 5′-TG GTGAAGACGCCAGTGGA-3′.

2.3. Cell culture and siRNA transfection

PrEC was provided by Lonza, Basel, Switzerland. LNCaP, DU145, and PC3 were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. C4-2 and C4-2B were provided by MD Anderson Cancer Center, TX, USA. 22Rv1 was provided by Y. Nakai. PrEC was cultured using Reagent Pack (Lonza) at 37 °C under 5% CO_2. Prostate cancer cells (LNCaP, 22Rv1, C4-2, C4-2B, DU145, and PC3) were cultured in RPMI1640 (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2.

For knockdown in 22Rv1 cells, transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) with antibiotic-free medium. siRNAs for PAX2 (10620318), MRGBP (124238), CNPY2 (115810) and MEP1A (106470) were purchased by Invitrogen.

2.4. Cell growth assay

22Rv1 cells were transfected with siRNAs using Lipofectamine RNAiMAX. Three days after transfection, 5000 cells were plated in each well of 96-well plates and cultured for 1–3 days. Living cells were counted using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and a micro plate reader (VERSA Max; Molecular Devices, Tokyo, Japan) [13].

2.5. Colony formation assay

22Rv1 cells were transfected with siRNAs using Lipofectamine RNAi MAX for 3 days. Then, 1×10^3 cells were plated in each well of a 6-well plate and cultured for two weeks. At the end of the assay, the cells were stained with Giemsa, and colonies were observed by eye [13].

2.6. Invasion assay

Using uncoated and Matrigel-coated Transwell inserts (Becton Dickinson, NJ, USA), invasion assays were performed according to the manufacturer's instructions. Three days after siRNA transfection, 2.5×10^5 living cells were added to the inserts for invasion assays. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Using the Diff-Quik™ staining kit (Sysmex, Hyogo, Japan), cells that invaded the inserts were stained, and the total stained cell number was counted [14].

2.7. Statistical analysis

Statistical analyses were carried out by *t*-test and Chi-square test as appropriate. All data are reported as means ± SD. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Cell growth and migration of secondary cells are regulated by homologues of human metastasis regulators

To perform a genetic screen for regulators of human prostate cancer progression using secondary cells of the *Drosophila* accessory gland, we used the *esg^{ts} Flp-Out* control system carrying a transgene encoding nuclear-localized GFP (GFP_{nls}), which lies downstream of the *Upstream Activation Sequence* (*UAS*) [3,11]. Using this system, we monitored secondary cell events by GFP_{nls} fluorescence. Secondary cell growth and migration are known to occur after mating [3]. We observed migration of secondary cells in 10-day-old males mated with a single virgin female as GFP_{nls} fluorescence under *esg^{ts} Flp-Out* control (Fig. 1A) [3]. Next, to investigate whether knockdown of the fly homologues of human metastatic regulators affects behavior of secondary cells, we knocked-down several genes in secondary cells using *UAS-RNAi* lines (Table 1). We previously suggested that human PAX2 promoted cell growth and migration of prostate cancer cells [14]. *Drosophila paired* (*prd*) is a homologue of human PAX2, a human metastasis regulator, and is known to be required for accessory gland development [15]. We tested whether knockdown of *prd* affects secondary cell behavior in 10-day-old males under *esg^{ts} Flp-Out* control. GFP-labeled secondary cells were barely detected in some of the *prd* knockdown males (Fig. 1B). We also examined the cellular behaviors of secondary cells in knockdown lines of well-known regulators of human prostate cancer progression. Mammalian N-cadherin (N-cad) commonly increases migration of cancer cells [16]. The number of GFP-labeled secondary cells was decreased in some *N-cad* knockdown males (Fig. 1B). Mammalian E-cadherin (E-cad) is down-regulated in invasive tumors, and this may have led to tumor progression [16]. In some *E-cad* knockdown males, secondary cell migration was increased (Fig. 1B). These results suggested that cell growth and migration of secondary cells are regulated by the *Drosophila* homologues of human cancer progression regulators. This system might be useful for genetic screening for regulators of human prostate cancer progression.

3.2. Genetic screening for human prostate cancer progression regulators in the Drosophila accessory gland

Next, to identify novel genes regulating secondary cell behavior upon mating, we performed genetic screening for regulators of cell growth or migration in secondary cells of mated 10-day-old males under *esg^{ts} Flp-Out* control using RNAi strains from NIG-FLY (Table 1 and 25 candidate factors specifically expressed at high levels in the accessory gland were selected by reference to ***modENCODE***

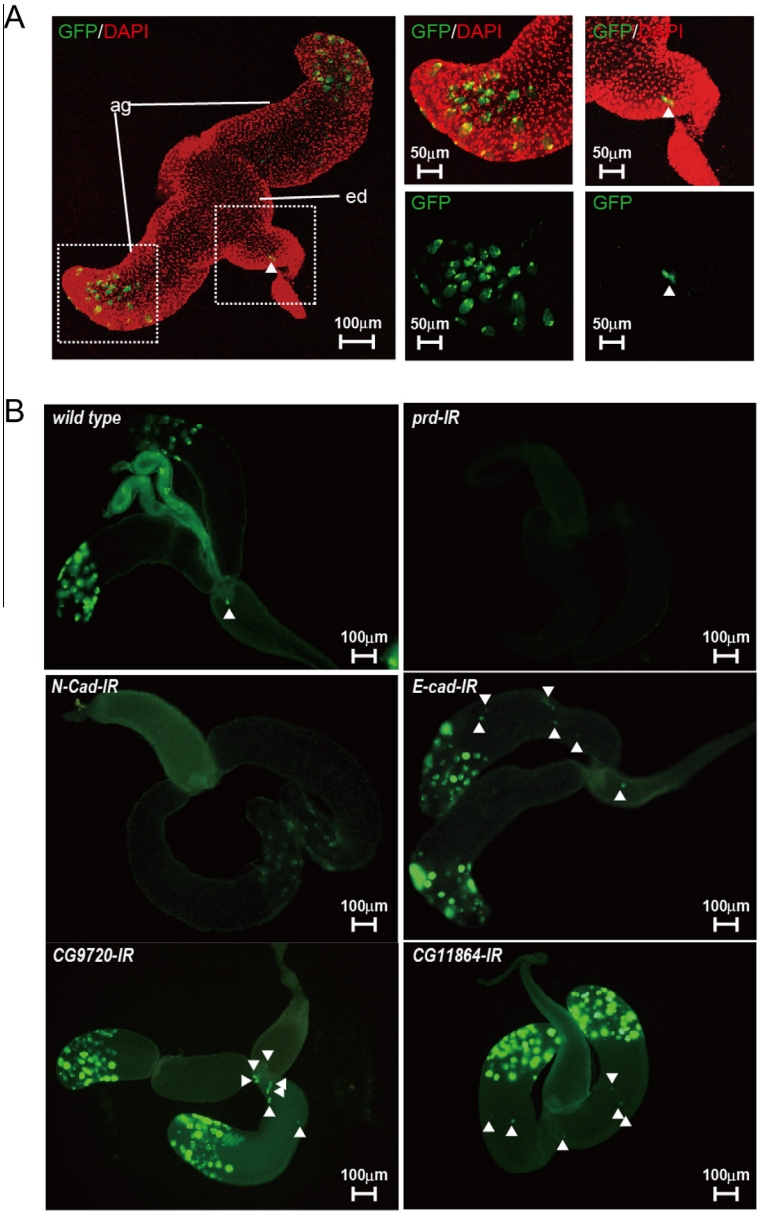

Fig. 1. Secondary cell growth and migration are controlled by *prd*, *N-cad* and *E-cad*. (A) Migration of secondary cells in mated males. Two accessory glands (**ag**) and ejaculatory duct (**ed**) of a 10-day-old male (esg^{ts} *Flp-Out* strain) mated with a single virgin female were imaged using confocal microscopy. Nuclear GFP (green) was expressed in secondary cells at the distal tips of the glands under *Flp-Out* control. In about 35% of mated 10-day-old *wild-type* males, a migrated secondary cell was observed in the ejaculatory duct (arrowheads). Boxes in the left panels are magnified in the right panels. Cell nuclei were stained with DAPI (red). (B) Cell growth and migration of secondary cells were altered in each knocked-down male. Accessory glands of mated 10-day-old males were observed using fluorescence microscopy. Migration of GFP_{nls} (green) is indicated by arrowheads.

high-throughput RNA-seq data [17]). As a result, the following observations were made: (i) cell number and (ii) migration of GFP-labeled secondary cells was altered in some RNAi-expressing knockdown flies (Table 1, Fig. 1B and Fig. 2A). Moreover, knockdown of *MrgBP*, *seele* (*sel*) and *CG11864* altered secondary cell behavior more significantly than did knockdown of other genes. Decrease in the number of GFP-positive secondary cells was observed in *MrgBP* and *sel* knockdown flies (Fig. 2A), while migration of secondary cells was elevated in some *CG11864* knockdown flies (Fig. 2A). To assess whether these three genes are involved in adult accessory gland development, the expression levels of these genes were quantified at different developmental stages. By quantitative qPCR analysis, *prd* was found to be expressed during accessory gland development, with expression slightly decreasing with progression of development in adult accessory glands (Fig. 2B). *MrgBP*, *sel* and *CG11864* were also expressed at each developmental stage, and their levels appeared to decrease over time (Fig. 2B). The expression profiles of these three genes were similar to that of *prd*. These data suggested that MrgBP, CG11864 and sel regulate cell growth or migration of secondary cells during accessory gland development.

3.3. Novel genes, identified by genetic screening using Drosophila, regulate invasion and cell growth of human prostate cancer cells

Drosophila MrgBP, *sel* and *CG11864* show high homology to human MRGBP, CNPY2 and MEP1A, respectively. We examined the expression levels of these three genes in several human

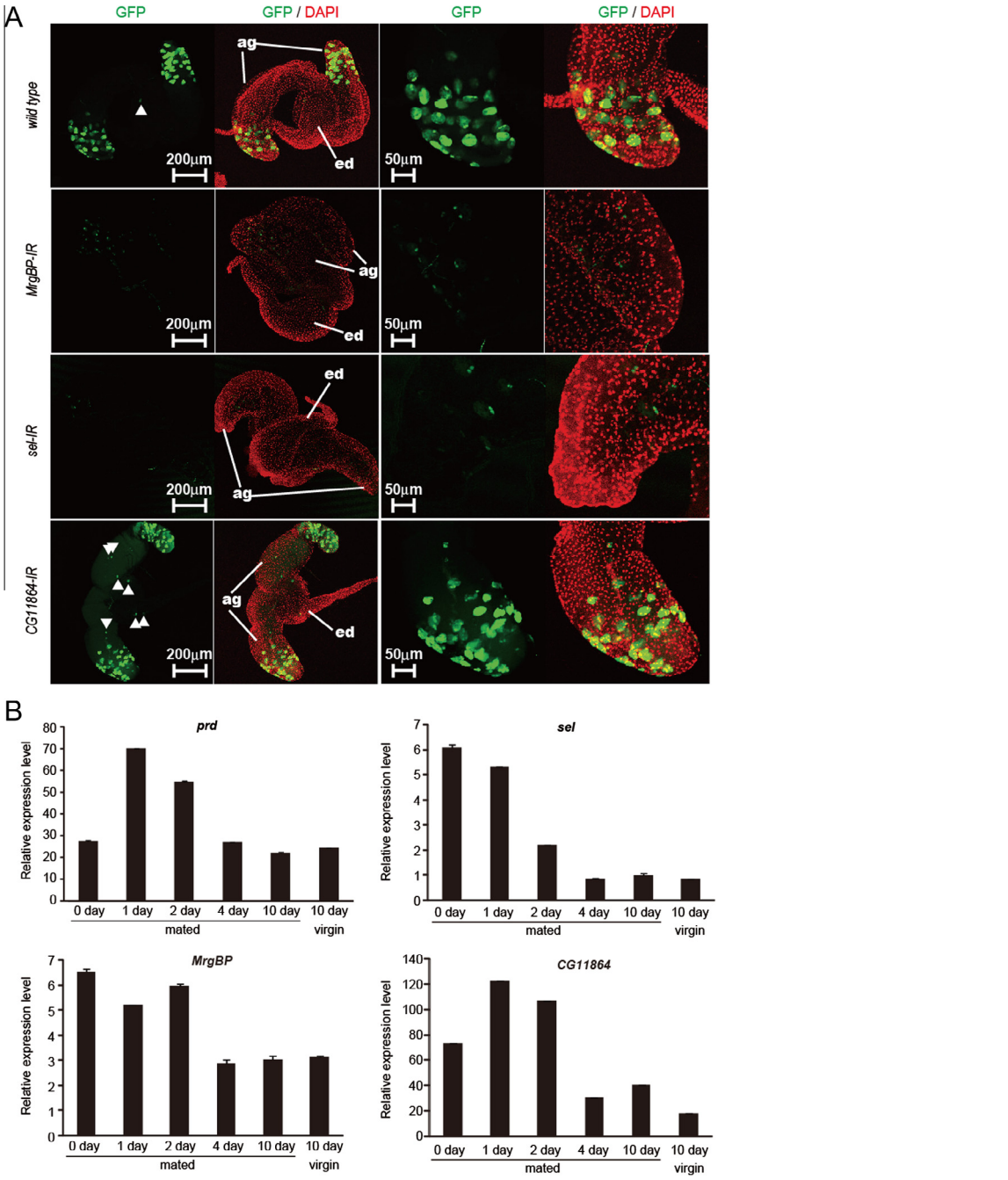

Fig. 2. *dMrgBP*, *sel* and *CG11864* regulate secondary cell growth and migration during development of the adult male accessory gland. (A) Secondary cell growth and migration in knockdown males. Accessory glands of mated 10-day-old males were imaged using confocal microscopy (green, GFP_{nls}; red, DAPI; left two panels). The distal tips of accessory glands in the left panels are magnified in the right two panels. Each gene was knocked down using the *Flp-Out* system. Migrated secondary cells are indicated by arrowheads. (B) Expression level of metastatic candidate genes during development of adult accessory glands. Total RNA was extracted from a pair of accessory glands and an ejaculatory duct, reverse-transcribed, and quantified using qPCR. Each measurement shows the average values of more than three independent measurements, which were normalized to the expression levels of *rp49* mRNA.

prostate cancer cells. PrEC cells are normal human epithelial cells. DU145 and PC3 cells are human prostate carcinoma cells derived from brain and bone metastases, respectively [18,19]. DU145 and PC3 cells do not respond to androgen [20]. LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells derived from a lymph node metastasis [21]. C4-2 and C4-2B are derived from LNCaP cells, and are androgen-insensitive and AR positive [22]. 22Rv1 cells are human prostate carcinoma epithelial cells derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CER22 xenograft [23]. mRNA expression levels in these prostate-derived cell lines were quantified by qPCR analysis. The results showed that MRGBP, CNPY2 and MEP1A were expressed in each prostate cancer cell (Fig. 3A). Furthermore, these three factors were more highly expressed in 22Rv1 cells than in other cells (Fig. 3A). To assess whether these factors regulate prostate cancer cell growth and cell invasion, we performed cell growth, colony formation and invasion assays using 22Rv1 cells that were transfected with each siRNA (Fig. 3B). Cell growth was inhibited by knockdown of CNPY2, but was not inhibited by knockdown of either MRGBP or MEP1A in 22Rv1 cells (Fig. 3C). Colony formation by 22Rv1 cells was also inhibited by knockdown of CNPY2, but not

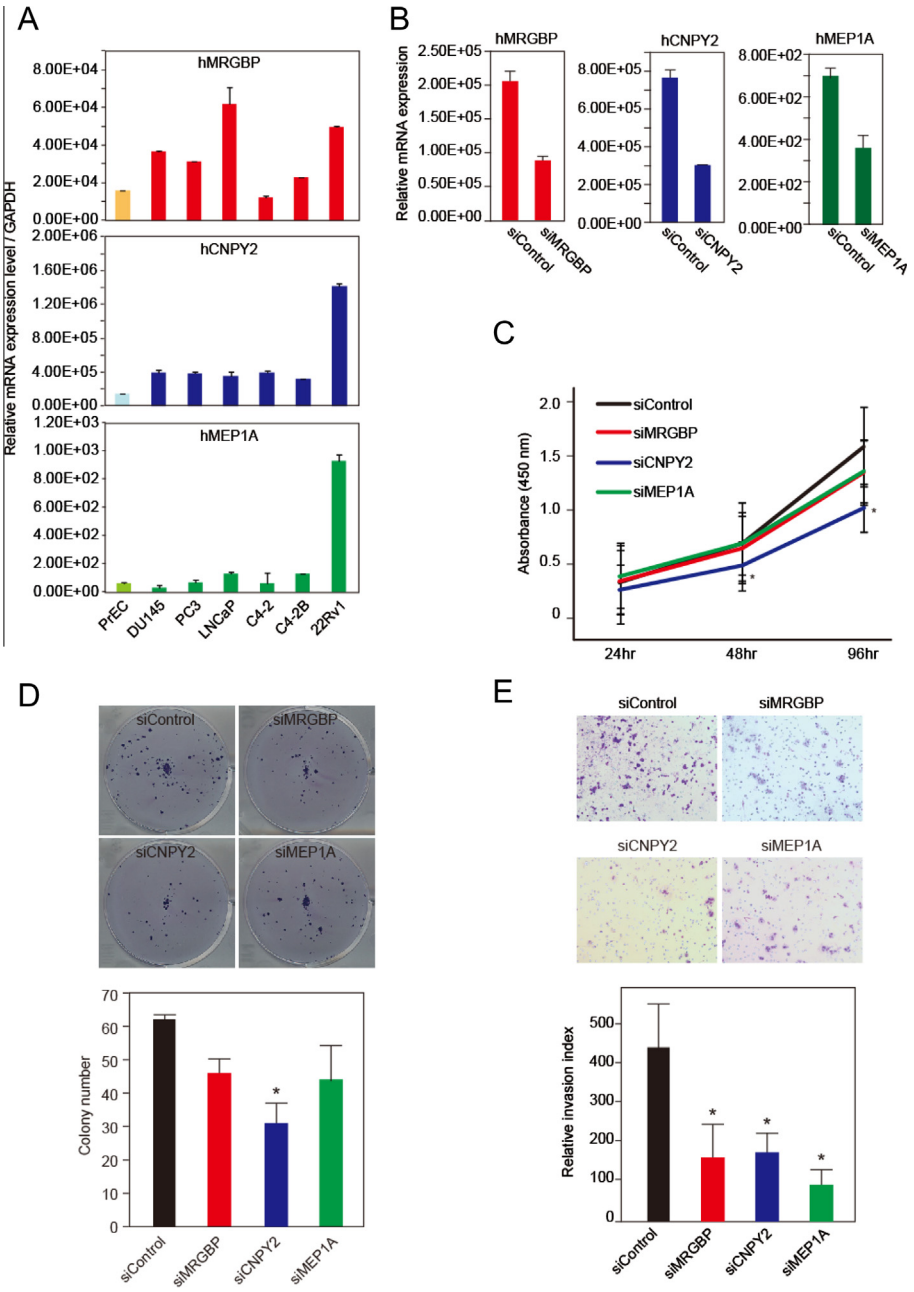

Fig. 3. hMRGBP, hCNPY2 and hMEP1A enhance invasion activity in 22Rv1 cells. (A) hMRGBP, hCNPY2 and hMEP1A were expressed in prostate cancer cells. The mRNA expression levels of these three genes were quantified using qPCR. Shown are the average values of three independent measurements, which were normalized to the expression levels of *GAPDH* mRNA. (B) Expression levels in knocked-down 22Rv1 cells. Total RNA was extracted from 22Rv1 cells transfected with each siRNA for 3 days. (C) Cell growth of 22Rv1 cells transfected with siRNAs. Cells were plated in 96-well plates at 3 days after siRNA transfection (0 h). Cell numbers were determined using the CCK-8 kit and are reported as absorbance at 450 nm ($n = 4$). $^{*}P < 0.05$. (D) Colony formation assay using knocked-down 22Rv1 cells. Cells were transfected with siRNAs and then cultured for 2 weeks (upper panel). Colonies in each dish ($n = 3$) were visually counted and numbers presented using a column graph (bottom panel). $^{*}P < 0.05$. (E) Invasion assay using 22Rv1 cells transfected with siRNAs. Invasive cells were visualized with purple staining (upper panel). The number of invasive cells was counted ($n = 3$; bottom panel). Invasion indexes are shown as relative to control inserts.

inhibited by knockdown of either MRGBP or MEP1A (Fig. 3D). The invasion activity of 22Rv1 cells was inhibited by knockdown of all three genes (Fig. 3E). These results suggested that these genes are positive regulators of human prostate cancer progression through the promotion of cancer cell invasion.

4. Discussion

Most prostate cancer patients initially benefit from androgen deprivation therapy since activation of the androgen–androgen receptor (AR) pathway promotes prostate cancer progression. In later stages, the cancer becomes treatment resistant and is called castration-resistant prostate cancer (CRPC). Metastatic CRPC (mCRPC) is notably associated with a poor prognosis and short-term survival. Currently, an effective therapy for CRPC has not been established. Some reports have suggested that androgen-AR pathways are required for CRPC progression [24], while others have suggested that signaling pathways other than the androgen-AR pathway are activated in CRPC. The finding of novel regulatory factors activated in CRPC model cells is beneficial for understanding the mechanisms of prostate cancer development.

Screening systems using model organs during development are thought to be helpful tools for studying cancer progression mechanisms because many mechanisms underlying cancer progression are thought to underlie organ development, as well. In the present study, we showed that a screening system using the secondary cells of the *Drosophila* accessory gland, which is homologous to the human prostate, is useful for investigating the regulatory mechanisms of cell growth and invasion of human prostate cancer cells. Cell growth of secondary cells upon mating was suppressed by down-regulation of the homologues of metastatic regulators of human prostate cancer PAX2 and N-cadherin. In addition, migration of secondary cells was promoted by down-regulation of E-cadherin. We identified several novel candidate genes regulating invasion and cell growth of secondary cells. We found that the human homologues of three of the *Drosophila* candidate genes regulated invasion and growth of human prostate cancer cells. Investigation of the functions of the human homologues of other candidate genes is a matter of future work.

We found three novel genes that promoted invasion of human prostate cancer cells. Human MRGBP/URCC4, a *Drosophila* MrgBP homolog, is a subunit of the TRRAP/TIP60-containing histone acetyltransferase complex [25]. It has been reported that MRGBP contributes to the development of colorectal cancer and cutaneous squamous cell carcinoma [26,27]. Human CNPY2/MSAP, a *Drosophila* seele homolog, was identified as a positive regulator of neurite outgrowth [28] and migration of C6 glioma cells [29]. Human MEP1A, a *Drosophila* CG11864 homolog, is a metalloprotease [30]. A previous report showed that MEP1A functioned in colorectal cancer [31]. All three of these genes promoted invasion activity in a human prostate cancer model cell line, 22Rv1 (Fig. 3). CNPY2 also promoted cell growth and colony formation activity in 22Rv1 cells (Fig. 3). The results of our study suggested that while three *Drosophila* genes are negative regulator of migration, the human homologues are positive regulator. One possible explanation for this contradiction may be the existence of other MEP family proteins, such as MEP1B [32]. In mammals, MEP1A knockdown may induce MEP1B overexpression. Study of other MEP family members may be needed for understanding of MEP1A function in cancer progression. The molecular functions of these three genes have not been uncovered in prostate cancer progression. Further studies on these newly identified factors may provide new insights into the molecular mechanisms of prostate cancer progression. The androgen-AR pathway does not exist in *Drosophila*; therefore, the genes uncovered in this study may be therapeutic targets for CRPC.

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