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14. ABSTRACT

In the United States there are over 40000 new endometrial cancers detected annually. Fortunately many of these women will survive past 5 years following standard treatments of surgery and surgery combined with radiation or chemotherapy. However, about 8000 women are not as fortunate and will succumb to their disease despite good clinical care. Data from our lab and others has documented that endometrial cancers may share some similarities or molecular signatures with the traditional pathologic and clinical definitions of these tumors. However, these cancers are in reality quite individual, and those certain cancers that follow an aggressive clinical course are likely the result of underlying molecular changes that account for these cancers behaviors.

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Introduction.

In the United States there are over 40000 new endometrial cancers detected annually. Fortunately many of these women will survive past 5 years following standard treatments of surgery and surgery combined with radiation or chemotherapy. However, about 8000 women are not as fortunate and will succumb to their disease despite good clinical care. Data from our lab and others has documented that endometrial cancers may share some similarities or molecular signatures with the traditional pathologic and clinical definitions of these tumors. However, these cancers are in reality quite individual, and those certain cancers that follow an aggressive clinical course are likely the result of underlying molecular changes that account for these cancers behaviors.

PROGRAM II: Molecular Profiling of Gynecologic Cancers

Project Leaders: John Risinger, PhD (Michigan State University), Thomas Conrads, PhD (Women's Health Integrated Research Center at Inova Health Systems)

To evaluate the accuracy of genomic, transcriptomic, proteomic, and metabolomic techniques as well as other future methods to develop and ultimately validate molecular profiles, in tissues, blood, serum, urine and cells from patients with gynecologic conditions including cancers, that can improve our understanding of the molecular alterations associated with gynecologic malignancies and benign gynecologic conditions; and/or facilitate optimal disease detection and prevention, delivery of care or development of more effective treatments. Program II is currently focusing on proteomic and genomic profiles / markers associated with or predictive of metatstasis, disease recurrence/progression as well as drug resistance/sensitivity.

MSU: MSU scientists will participate in gynecologic research in collaboration with the GYN-COE. As such the site PI, Dr. John Risinger will be involved in many aspects of the award including regular participation in planning and experimental design, interpretation and progress in conjunction with the Executive Team at the GYN-COE (Drs. Maxwell, Hamilton, Conrads and Darcy). MSU scientists will principally be involved in the molecular profiling project described in detail in the appendix of the application. Dr. Risinger and his team will be responsible for the experiments summarized below. Dr. Risinger and his team will be involved in the interpretation and analysis of gene expression array data as well as next generation expression and sequence results through-out the duration of the grant. They will work in conjunction with the GYN-COE to finalize specific gene targets that merit further experimental validation of examination in pre-clinical models of endometrial cancer.

MSU will also function in providing his expertise in the statistical confirmation and identification of biomarkers associated with factors related to clinical outcomes of uterine and ovarian cancer. These will include a comprehensive evaluation and analysis of mRNA and miRNA gene expression data associated with endometrial and ovarian cancers as well as their suspected normal tissues of origin. He will provide analysis that will help guide the design of experiments related to the biologic function of identified molecules. This will be achieved by providing analyses directed towards functional

significance through the use of integrated bioinformatic and systems biology approaches. These approaches will help to classify genes and their associated networks. Dr. Risinger will interact and consult with the GYN-COE and its other bioinformatics and systems biologists to achieve state of the art science related to biomarker discovery and biologic significance. Importantly he will also serve in the design and analysis of experiments related to cell function such that these studies are designed with appropriate statistical power and scientific rigor.

MSU will also perform experimental validation of mRNAs target identified by comparison of metastatic and non-metastatic endometrial cancers (SA1) and recurrent and non-recurrent stage 1 endometrial cancers (SA3)and novel mutated or expression targets identified in SA3 as described in project 2. RNAs necessary for validation will be received from Women's Health Integrated Research Center (WHIRC) at Inova Health Systems in Annandale VA for the GYN-COE following microdissection and will be processed to cDNA. cDNA samples will be examined using highly specific quantitative RT-PCR for identified mRNAs and ncRNAs. Some validated biomarkers will be assessed for biologic function. Specifically whether *in vitro* modulation by gene knock-down and add- back can affect neoplastic endpoints described in project 2.

The purpose of this work is to increase our understanding of the molecular aberrations associated with endometrial and ovarian carcinogenesis and the biologic mechanism(s) associated with these defects and their relation to clinical and pathologic factors.

Body

The work is summarized.

Cell line models : Significant effort was undertaken to characterize specific cell based reagents targeted for use in future gene manipulation studies. These studies were designed to substantiate previously described characteristics of known models and also to characterize newly developed models for which cell based assay behavior was unknown or incomplete. Specifically gene knock-down and add back studies will require normal endometrial cells that are immortalized without oncogenes, retain ovarian steroid response and are non-tumorigenic in immune compromised mice. The best model described in the literature that satisfies these criteria is the EEC-TERT cell line(1). Recently unpublished data obtained by MSU collaborators at Northwestern suggest these cells were tumorigenic and also metastatic. These cells were obtained from Dr. Fazleabas collection of cell lines. The Risinger lab at MSU is utilizing EEC-TERT cells obtained directly from the originators Drs Klonisch. The laboratory has recently obtained 12z cells derived from endometriosis a precursor of gyn malignancy. Studies will also require cells with PPP2R1A mutation(2-5). We identified PPP2R1A mutation in ACI-89 derived from an ovarian clear cell carcinoma and ACI-158 derived from a uterine serous carcinoma(2). Both these cells were developed by Dr. Risinger.

Function and clinical studies of ARID1A, ARID1B and PPP2R1A: Next generation sequencing efforts targeted to ovarian clear cell cancers identified mutations in the swi/snf chromatin remodeling gene ARID1A and in the structural component gene PPP2R1A of the PP2A phosphatase(5, 6). Mutations of ARID1A were confirmed to be common in endometrioid and clear cell cancer of ovary. Mutation and loss of expression were also noted in a high frequency of uterine carcinomas(7, 8). PPP2R1A mutations were found to frequently occur in the serous type of uterine cancer(2-4). The function of these genes in malignancy is unclear.

A major focus of effort in coming months will be to examine changes to cell phenotypes when the *ARID1A/B* or *PPP2R1A* genes are manipulated in appropriate uterine and ovarian cell line models. Furthermore cellular consequences in terms of mRNA and protein levels will be examined using models developed in this quarter. Furthermore effects on the phosphoproteome will be examined utilizing models developed in this quarter.

Race as a surrogate for poor outcome: A racial disparity in incidence and survival exists for many human cancers. Understanding and addressing the reasons for these disparities is critical for reducing cancer burdens. A well described disparity in both incidence and survival outcome exists for endometrial cancer. Population based studies using data from both the National Cancer Data Base and Surveillance, Epidemiology, and End Results (SEER) have consistently shown that Caucasians (CA) are more likely to develop this cancer than are African Americans (AA) even after controlling for hysterectomy(9-11). However, African American women are about two times more likely to die from their disease than are Caucasians(11, 12). The reasons underlying these disparities are complex and include social, cultural and biologic factors(13). Several molecular genetic studies have identified differences in the prevalence of certain gene mutations or expression aberrations in cancers from AA and CA women. For example, the TP53 tumor suppressor gene is mutated more frequently in endometrial cancers from AA women(14). Similarly the HER2/Neu oncogene is more frequently up-regulated in endometrial cancer from AA women(15). In addition, a specific chromosome gain on chromosome one is more frequent in endometrial cancers from AA women(16). Epigenetic methylation of the ribosomal DNA is also less prevalent in endometrial cancers from AA women(17). TP53, rDNA and HER2 events are tied to more aggressive behavior and adverse outcome in endometrial cancer, suggesting they may be responsible in part for the outcomes disparity for AA women. In contrast, the PTEN tumor suppressor gene is more frequently mutated in cancers from CA and is associated with a more favorable outcome(18).

Recently, we performed transcript expression studies in endometrial cancers(19-22). These studies showed distinct expression related to histologic type as well as identifying transcripts associated with microsatellite instability. We performed an initial study in which we specifically compared stage and grade matched endometrial cancers from AA and CA patients to identify whether distinct transcripts were associated with the race of the patient and, if so, whether these could serve as candidates for diagnosis, prognosis or intervention. A previous study using a very similar study design found no global differences between AA and CA but did identify a small sub-set of genes differentially expressed. Although global differences between the two groups are not evident in our first study, we do identify a

number of statistically differentially expressed transcripts, a subset of which were validated by quantitative real time PCR. Among those validated was that encoded by the gene for phosphoserine phosphatase like (PSPHL), which was found to be prominently expressed in endometrial cancer specimens from AA women and cloned an additional splice variant also preferentially expressed in AA women. The data from this initial study and the relevance of the PSHPL gene in endometrial cancer and other tissue was prepared for publication and submitted to Frontiers in Womens Cancers.

The COE prepared and profiled a much larger set of laser captured endometrial cancers than in our initial or Ferguson et al studies. MSU was asked to re-evaluate these data in terms of global expression changes and specific changes associated with patient and clinical demographics.

Acomplishments

Unique endometrial cancer cell reagents developed by Dr. Risinger at the Curtis and Elizabeth Anderson Cancer Institute in Savannah, GA were disseminated to COE and COE collaborative work groups. This included the National Cancer institute (NCI), Bethesda MD, Women's Health Integrated Research Center at Inova, Annandale VA, Roswell Park Cancer Institute, Roswell Park, NY and the Uniformed Services University of the Health Sciences, Bethesda MD.

The laboratory tested several endometrial cell models for presence of ovarian steroid receptors. Of these we examined ECC-1 cells obtained both from the originator Dr. Satyawaroop and from the ATCC. Both lineages of ECC-1 were shown to harbor ER and PR (Figure 1). In addition we examined two lineages of Ishikawa cells both directly obtained from the originator. Neither of these were shown to possess significant levels of receptor (Figure1). We also examined a panel of endometrial cancers established by our lab in Savannah Georgia. Of these only the ACI-181 endometrial carcinoma cell line expressed significant levels of ER and PR (Figure 1).The endometrial cell line EEC-TERT derived from normal glandular epithelial cells following hTERT immortalization was obtained from Dr Hombach-Klonisch. Cells were received both in Savannah and Michigan.   Both lineages of EEC-TERT were shown to express ER and PR.

EEC-TERT and ACI-181 cells were further tested for expected gene expression response following estrogen or progestin treatment to determine whether receptors that were present were functional. The cells are responsive to ovarian steroids in an expected manner as evidenced by increased TFF1 following estrogen administration(Figure 2). The EEC-TERT cells were proven to be non-tumorigenic in the flank of immune compromised Nu/Nu mice, whereas ACI-158 and ACI-181 are tumorigenic (Figure 3). The behavior of the cells in migration and invasion assays is still under study.

We established a collaboration with our former colleagues Olga Aprelikova and Mark Simpson at NCI to examine the effect of miRNAs on endometrial stromal cell effects on tumorigenicity of endometrial cancer. An offshoot of these studies involved the ACI-158 uterine serous carcinoma cell line. The cell

line is highly tumorigenic and especially migratory and invasive in boyden chamber assays. Furthermore metastasis arise spontaneously in lymph nodes and lungs following flank injection. Cells derived from lung metastasis and re-cultured are even more highly tumorigenic and exhibit more penetrant metastasis.

Three variants of these cells were examined using affymetrix based gene expression and mass spec spectral count proteomics. Preliminary analysis of these data suggests that significant numbers of gene transcript changes occur between the weakly metastatic parental cells and the highly penetrant metastatic variants. Gene expression data was of high quality (Figure 4). This data is further depicted in an unsupervised global analysis of transcripts(Figure 5). The separation of classes was driven by a relatively distinct set of diifferentially expressed transcripts(figure 6). Detailed lists of transcripts and proteins are provided in supplemental files 1 and 2 and their overlap in supplemental file 3. Furthermore a preliminary analysis of significant molecules was used to identify GO pathways relevant to the variant differences (supplemental file 4). The ACI-158 cell line and its metastatic behavior provides numerous opportunities to study characteristics of aggressive uterine serous carcinoma.

ACI 89 ovarian clear cell cancer and ACI-158 uterine serous carcinoma cells were found to harbor the recurring PPP2R1A mutations (Figure 7). ACI-89 does not form tumors in nude mice based on our previous studies. 20 other endometrial and ovarian cell lines were found to be wild-type at these hotspots(Supplemental File 5).

Lentiviral constructs were developed to express nothing (empty vector), wild type PPP2R1A, Codon 179 mutant and codon 183 mutant PPP2R1A. We constructed both tagged and untagged versions in pLenti6.3/V5-DEST(Figure 8). Expression of tagged versions can confirm the introduced protein among background of endogenous protein but the V5 tag may effect function therefore both versions were used in phenotypic studies.

Virus were generated and used to infect EEC-tert cells with empty vector, wild-type and both mutant versions of PPP2R1A. These cells were examined for proliferation, colony forming on plastic and anchorage independent growth. Virus were also prepared and used to infect empty vector and wild-type PPP2R1A in ACI-158 serous carcinoma cells which harbor an endogenous PPP2R1A mutation. Levels of wild-type message were measured by real-time PCR and increases in total PPP2R1A were noted in wild-type over-expressors . Increases in message levels were confirmed by immunoblots(Figure 9).

Introduction of mutant versions of PPP2R1A had no effect on the cell proliferation of EEC-TERT nor did it effect the migration of these cells or colony formation rates in soft agar (Figures 10 and 11). Introduction of wild type PPP2R1A into ACI-158 cells also had no effect on proliferation (Figure 12). RNAs and Protein lysates from these cells were sent to WHIRC and were examined by MASS-SPEC proteomics and AFFYMETRIX based gene transcript expression analysis in an attempt to uncover whether the introduction of mutants had any cellular effects. The quality of Affymetrix data was in general very good. One array EEC-tert-179mut replicate c had extremely high signal values and stood out as an outlier (Figure13). In general introduction of additional wild-type PPP2R1A into PPP2R1A mutant ACI-158 induced the most transcript changes. Most of these resulted in transcript increases

(Figure 14). Few changes were noted in EEC-TERT cells consistent with no observable cellular effects (Figure 15). These changes were reflected in unsupervised analysis which showed that each cell line and its manipulation was distinct from other cells (Figure 16). Full lists of altered transcripts are included in the supplemental file 6. Spectral counts of identified proteins were also examined in PPP2R1A manipulated cells supplemental file 7. Altered transcripts were characterized by gene ontology (supplemental file 8). Cell experiments were repeated with non-V5 tagged versions of the PPP2R1A. These data are annotated as supplemental file 8 a. Because these were done initially with a V5 tagged versions of PPP2R1A to differentiate introduced versions from endogenous versions the arrays have been repeated with untagged versions and data are forthcoming in the following month.

Cells will be examined for effects on the phosphoproteome which should be directly targeted by PP2A in the coming months.

22 endometrial and ovarian cancer cell lines were examined for ARID1A and ARID1B loss by immunoblotting (Figure 17). Models with ARID1A loss were identified similar to results of ARID1A from analysis on primary tumors suggesting the models will be relevant for study of ARID1A. A single truncation mutation of ARID1B was noted in 1 of 8 OCC in the supplemental section of their publication. Therefore we also examined ARID1B. We noted many cell lines without ARID1B in addition to loss of ARID1A. We further confirmed the nuclear location of ARID1A in these cells and demonstrated loss of both proteins in ACI-98 cells(Figure 18). Because the ARID genes are a key component of the chromatin remodeling complexes in cells and may contribute to epigenetic mechanism of gene expression and repression we also examined cells from a classic murine model of hormone induced uterine carcinoma(23).   Specifically we examined three uterine cancer cell lines established from CD-1 mice treated neonatally with DES(24). These DES induced cancer lines expressed abundant Arid1A and Arid1B (Figure 19).

The ARID1A and ARID1B genes are very large and will require a concentrated sequencing effort to catalogue the exact defects in the human gynecologic cancer cell lines lacking protein. Sequencing efforts targeted at AN3CA and ACI-98 cells are ongoing. A hurdle has been the technical limitation of amplifying the extremely GC rich and large exon 1 of both ARID1A and ARID1B. We have however identified a truncation mutation of ARID1B in the AN3CA cell line. This represents the first endometrial cancer with such a mutation and explains the loss of protein in this reagent.

A full length ARID1A cDNA was obtained by WHIRC from Origene and transferred to MSU. The cDNA was reengineered for expression for expression of Myc/Flag tagged versions in a tetracycline inducible lentiviral system (Figure 20). We first re-engineered the recombination site of pLenti CMV/TO GFP-Zeo DEST to harbor att sites to flank unique AsiSI and Pme1 sites of the entry vector from Origene. The full length ARID1A was then moved to the reengineered pLenti CMV/TO GFP-Zeo DEST Tet/On vector following AsiSI and Pme1 restriction digestion and ligation. MSU

 has now received the ARID1B clone and it has been re-engineered similarly. We now have both ARID1A and ARID1B cloned in conventional lentiviral vectors and in the Tet/ON retroviral vector (Figure 20 and 21).

We have completed an initial quality assessment of the ARID1A vector. The ARID1A vector is capable of efficient expression as monitored by the GFP tag when introduced to 293 cells (Figure 22). The vector produces full length ARID1A as monitored by V5 and ARID1A immunoblotting , empty vector serves as control(Figure 23).

Our laboratory has obtained and transformed cloned necessary lentiviral packaging plasmids (pLP2, pLP/VSVG and pLP1). These have now been used to make initial lentiviral particles. A schematic for viral production is presented in Figure 24. The ARID1A and ARID1B genes are at the maximum size limits for viral particle production.   Our initial production indicates that lentivirus is produced from the vector but that we will need to increase its titer for high efficiency infections (Figure 25).   Stable TET repressor expressing versions of model cell lines are necessary for effective TET/ON viral production. We are currently generating these models according to schematic Figure 26. Cell line TOV21G (clear cell ovarian), AN3CA (metastatic endometrioid endometrial), ACI-98 (undifferentiated endometrial) will be transfected with mammalian expression vector pCMV/6TR and stable clones selected and screened by immunblot for TET repressor expression.

Neoplastic phenotypes of colony formation on plastic not dependent of TET/ON delivery are currently in progress in evaluate the tumor suppressive nature of ARID1A and B in these models.

We performed an initial assessment of the affect of five independent shRNA to ARID1A on the EEC-tert normal endometrial cells. The regions targeted are noted in Figure 27 and sequences of shRNA matching other published ARID1A studies noted. We noted knockdown of transcript and protein following infection (Figure 28). shRNA targeting ARID1A resulted in decreased proliferation in all five knockdown lines (Figure 29). These data are similar to knockdown of skin and pancreatic precursors (25). Despite sharing the same targeted sequences the data are in contrast to studies in ovary surface epithelial cells which were noted to increase their proliferation in response to ARID1A knockdown(26).

Race: We completed an initial assessment of race on endometrial cancers and prepared a manuscript which is in submission to "Frontiers in Womens Cancer".

In addition we received a set of Affymetrix plus 2.0 gene expression data from a larger set of endometrial cancers (rs2A).

Initial analysis of the data is complete. In our previous previous comparison of endometrial cancers between African American (AA) and Caucasian (C) we found 325 transcripts at p < 0.005 (RS1 data set) of which 66 were altered by 2-fold. These transcripts also have a geometric average signal greater than 100 either for AA or C cases. RS1 is based on 11 AA and 14 C cases. Paired T-test was used for this comparison using 14 pairs in which 3 AA cases were duplicated to make up 14 pairs. The p-values are over-estimates to this extent. However, pairing allowed histology, stage and grade effect compensation.

RS2A contains 25 AA and 25 C cases matched for histology and stage. There were 10 early stage and 15 advanced stage cases per race. C cases include 12 Serous and 13 Endometrioids while AA cases include 12 Endometrioid and 13 Serous cases. In these 50, there are 3 grade-1, 16 grade-2 and 31 grade-3 cases. These are LCM quality materials arrayed on HG-U133 Plus 2.0 chips. Class comparison was done

using two sample two-tailed T-tests. There were 44 transcripts at p < 0.005, altered by 2-fold with a geometric average signal > 100.

Endometrial expression of normals: Class comparison of AA vs. C using normal cases (3 AA and 3 C cases), identified 68 transcripts significant at p < 0.05 and 2-fold change. Interestingly, the only gene common to these two lists is PSPHL (CO9).

In the coming quarter we will assess this dataset further and examine the effect of other variables such as histology and grade with race to further investigate this set of data.

Figures (Provided in separate powerpoint file)

Supplemental files supplied separately

Note: Tumorigenicity studies did not utilize DoD monies
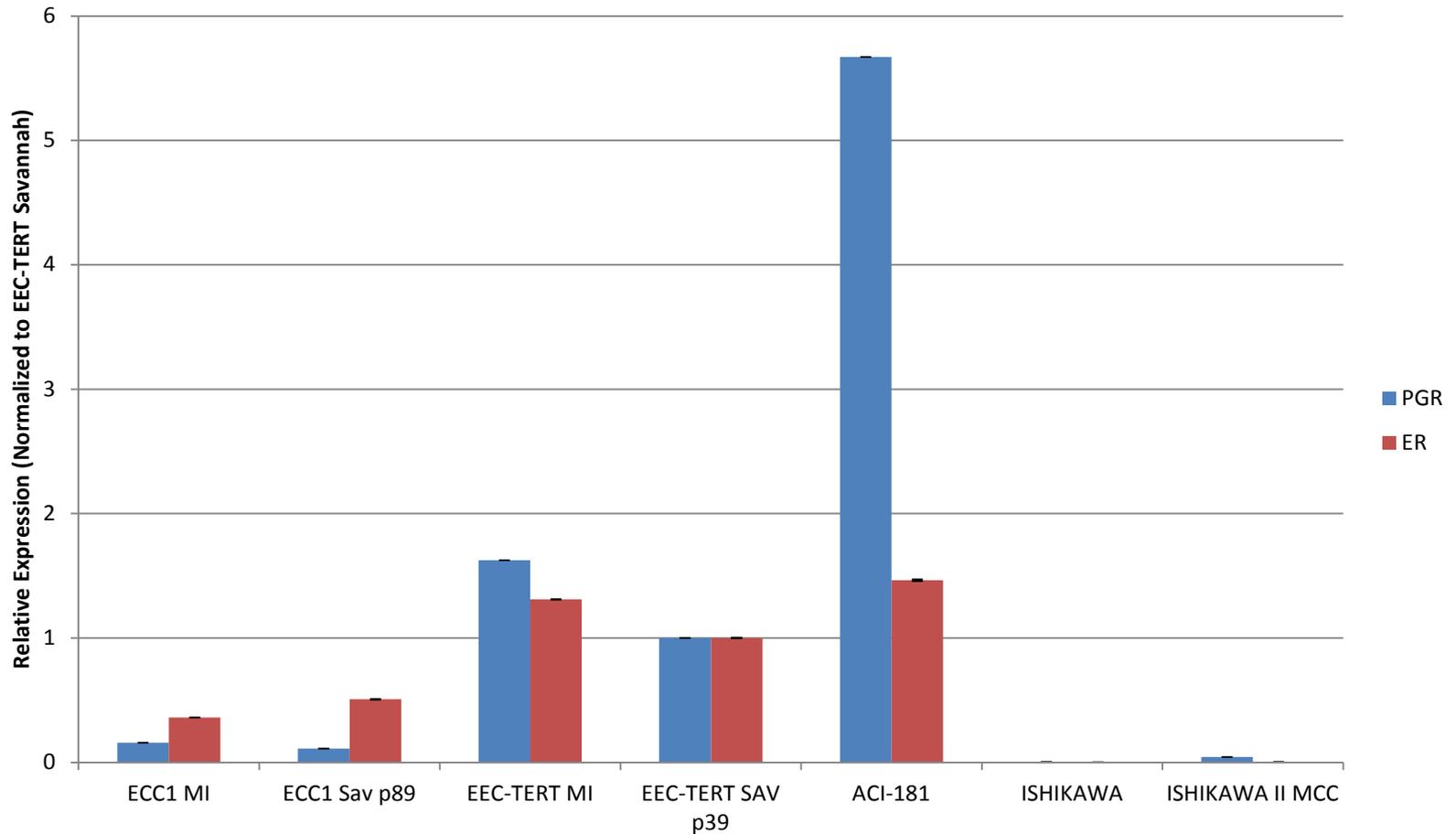
Figure 1. Ovarian steroid receptor levels in endometrial cell models. Real-time PCR of ER and PR levels compared to PPIA(cyclophillin).
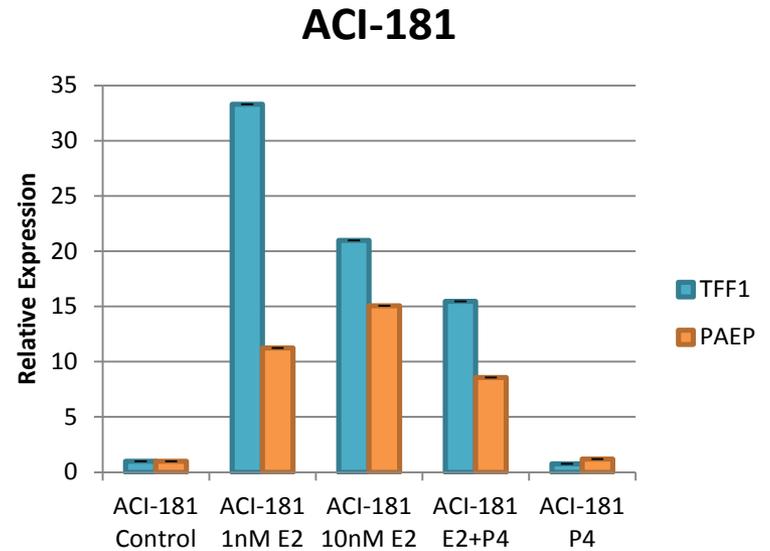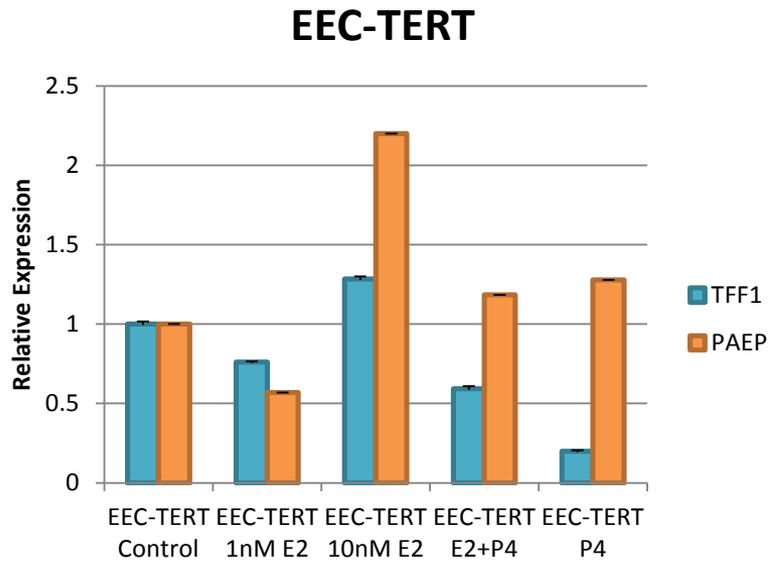
Figure 2. Gene expression following steroid administration. Relative expression of TFF1 and PAEP transcripts following addition of either estrogen , progestin or the combination in phenol free steroid free media.
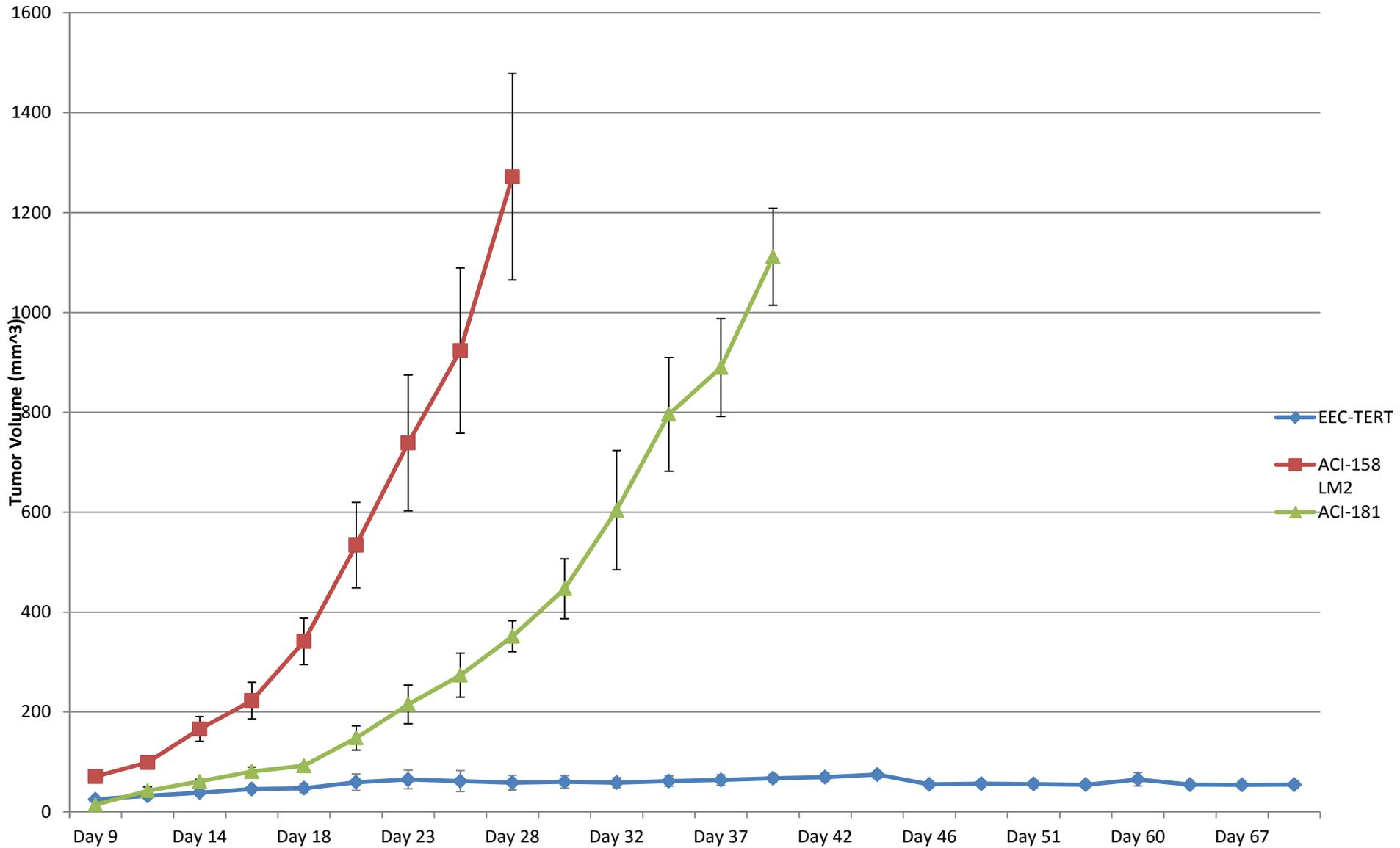
Figure 3. Tumorigenicity of endometrial cell line models.

Figure 4

Array Quality

Sample	File	Scale factor	% P + M	GAPDH-signal	GAPDH_3'/5' ratio
LUC1	JR1	2.07	45.4	24959	1.85
LUC2	JR2	2.38	42.3	25863	1.85
LUC3	JR3	2.59	45.5	31461	1.25
LUC_LU1	JR4	2.33	45.5	32786	1.13
LUC_LU2	JR5	2.14	45.5	30645	1.06
LUC_LU3	JR6	2.19	44.3	32444	1.06
LUC_LM21	JR7	2.20	45.2	31752	1.12
LUC_LM22	JR8	2.41	44.3	32582	1.09
LUC_LM23	JR9	2.29	44.8	31223	1.05
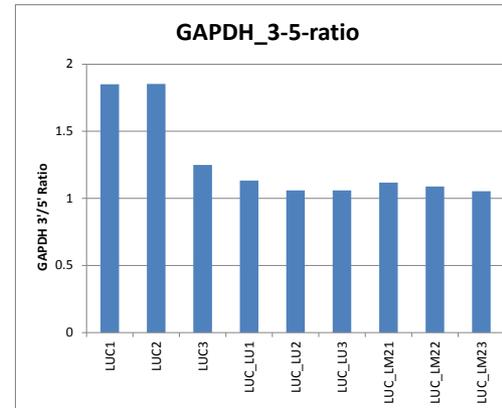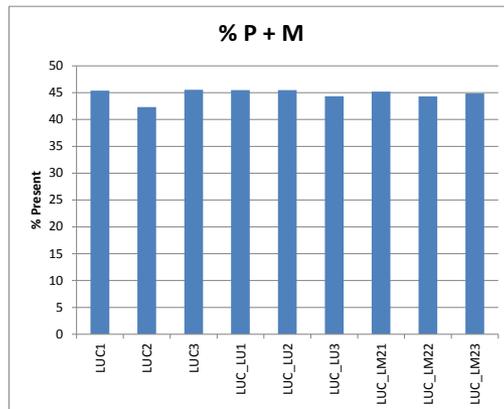
Global expression profiles of about 23.9 K transcripts found in at least half of the arrays
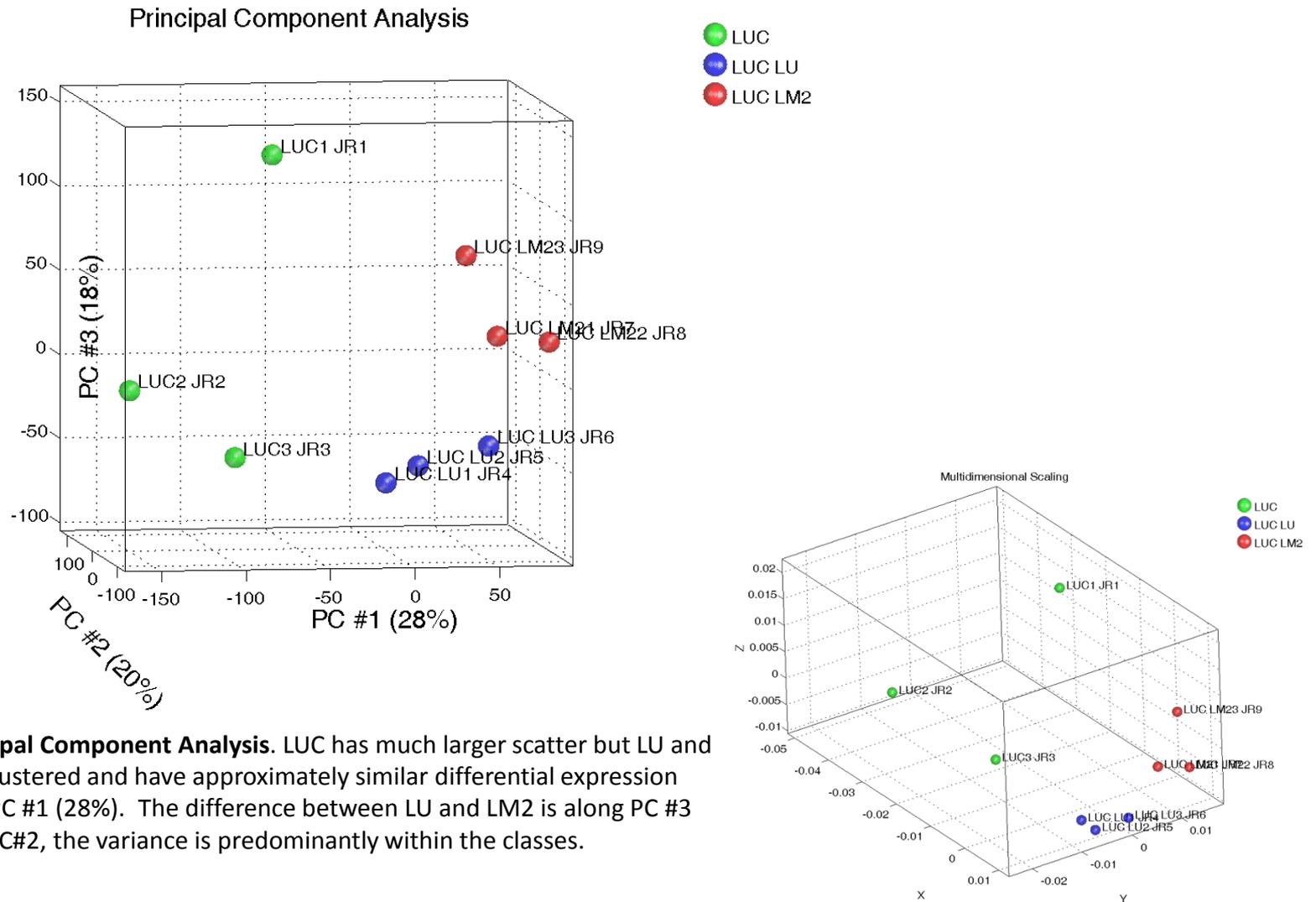
Figure 5. Principal Component Analysis. LUC has much larger scatter but LU and LM2 are well clustered and have approximately similar differential expression profiles along PC #1 (28%). The difference between LU and LM2 is along PC #3 (18%). Along PC#2, the variance is predominantly within the classes.

2-fold altered transcripts at p < 0.05, and one signal > 100
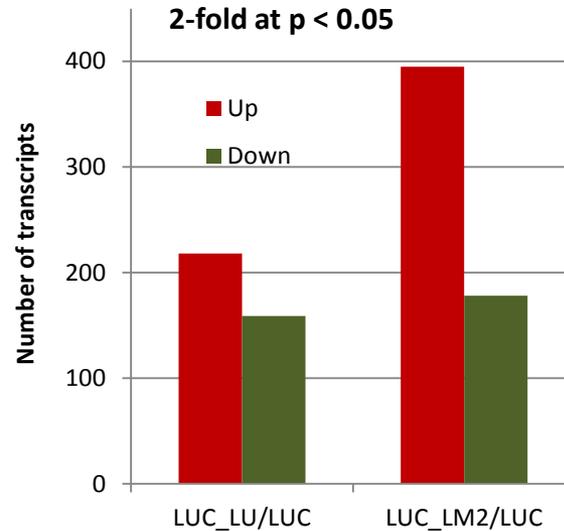
	Up	Down	Total
LUC_LU/LUC	218	159	377
LUC_LM2/LUC	395	178	573

Figure 6. General summary of the number of significant transcripts between variants.
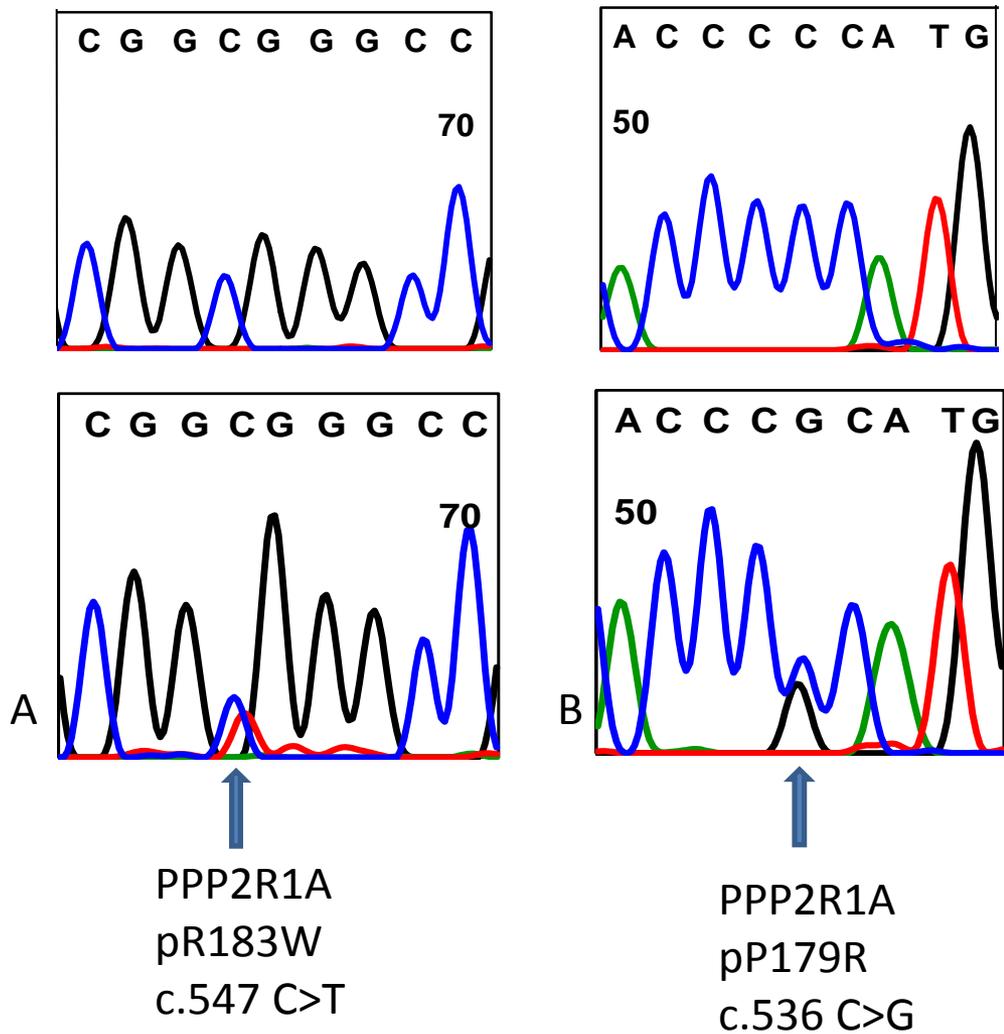
Fig. 7. Sequence chromatograms showing PPP2R1A mutations. The lower panels show the mutation region, and the upper panels show the wildtype. **A** is ovarian clear cell carcinoma cell line ACI-89 and **B** is uterine serous carcinoma cell line ACI-158.

A

PPP2R1A
pR183W
c.547 C>T

B

PPP2R1A
pP179R
c.536 C>G
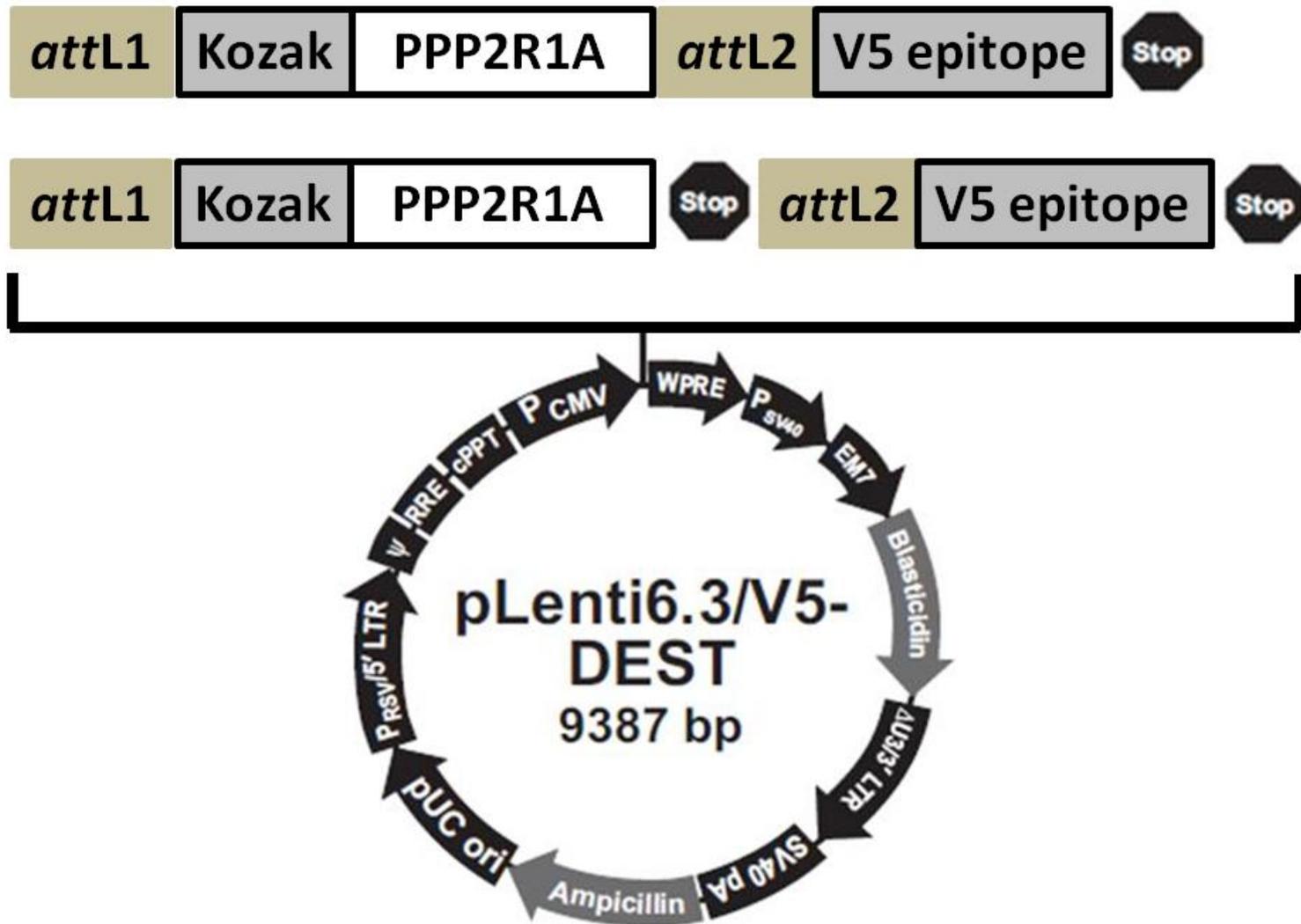
Figure 8. Lentiviral construct for PPP2R1A expression.

Figure 9. Immunoblot analysis of PPP2R1A
(A) C-terminal V5 tagged PPP2R1A were expressed in ACI-158 and EEC-TERT detected by anti V5 tag antibody. (B) Recombinant PPP2R1A (without V5 tag) detected by anti PPP2R1A antibody. WT : parental cell, vector : pLenti6.3 vector, PPP2R1A : wild type PPP2R1A, MUT183 : PPP2R1A mutant 183, MUT179 : PPP2R1A mutant 179.
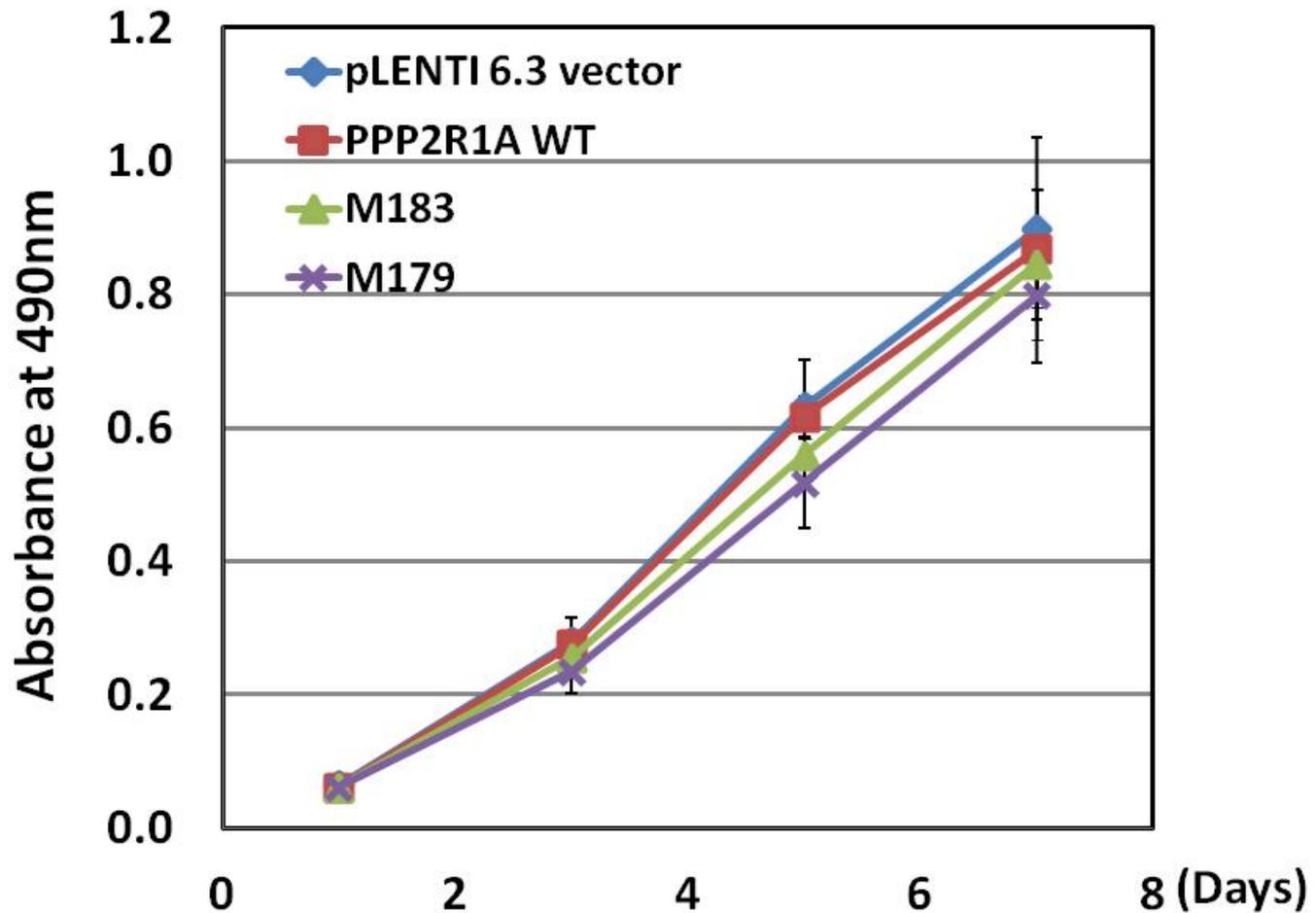
Figure 10. Proliferation of EEC-TERT following viral introduction of PPP2R1A mutants
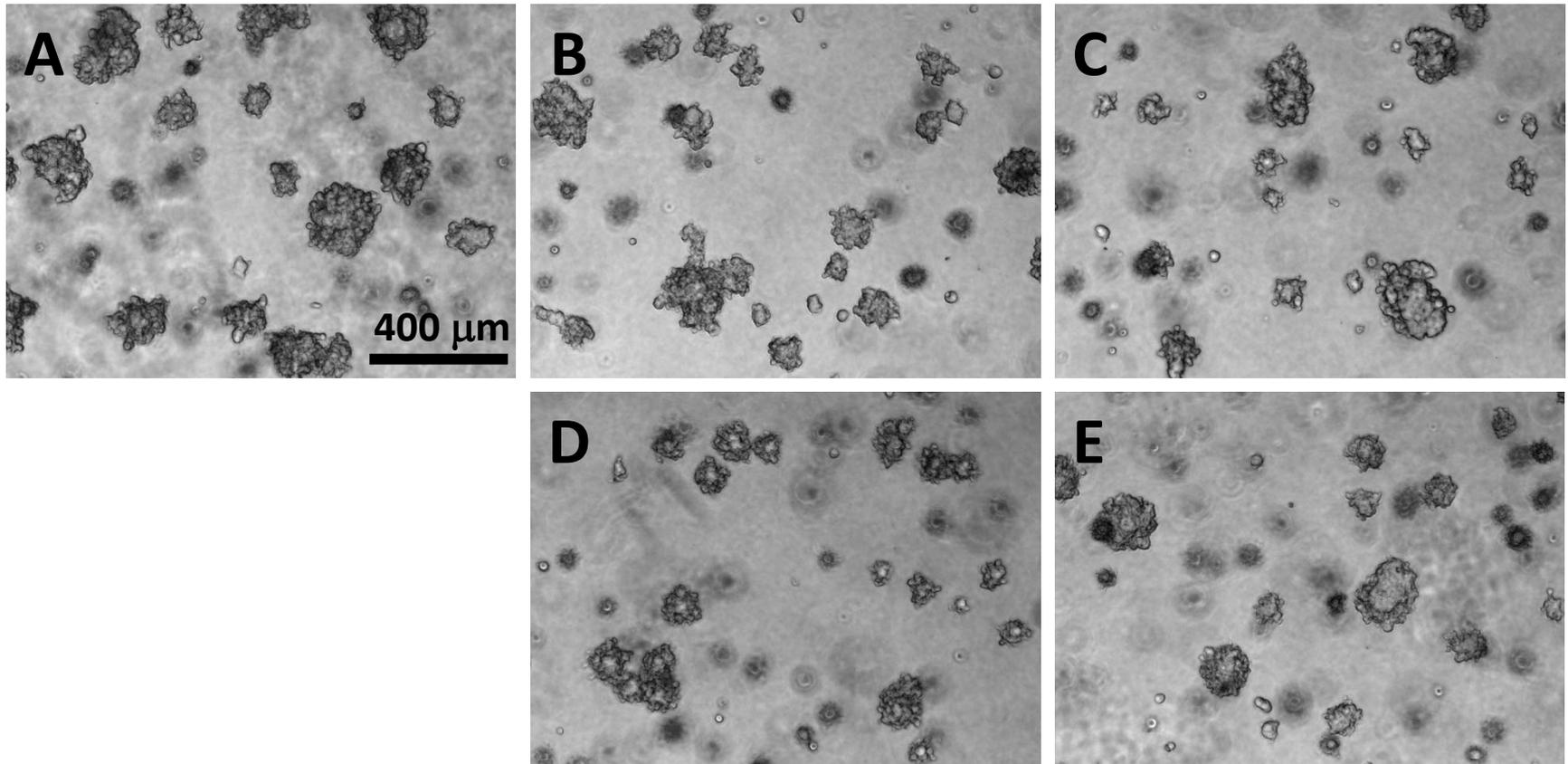
Figure 11. Soft agar colony formation assay of EEC-TERT

Cells (5×10^4) seeded to 6 well plate with 0.35% agarose / 10% FBS / DMEM F12 and cultured for 10 days in 37 ^{o}C and 5% CO_2. A : EEC-TERT, B : EEC-TERT/pLenti 6.3 vector, C : EEC-TERT/ PPP2R1A WT, D : EEC-TERT/ PPP2R1A M183, E : EEC-TERT/ PPP2R1A M179.
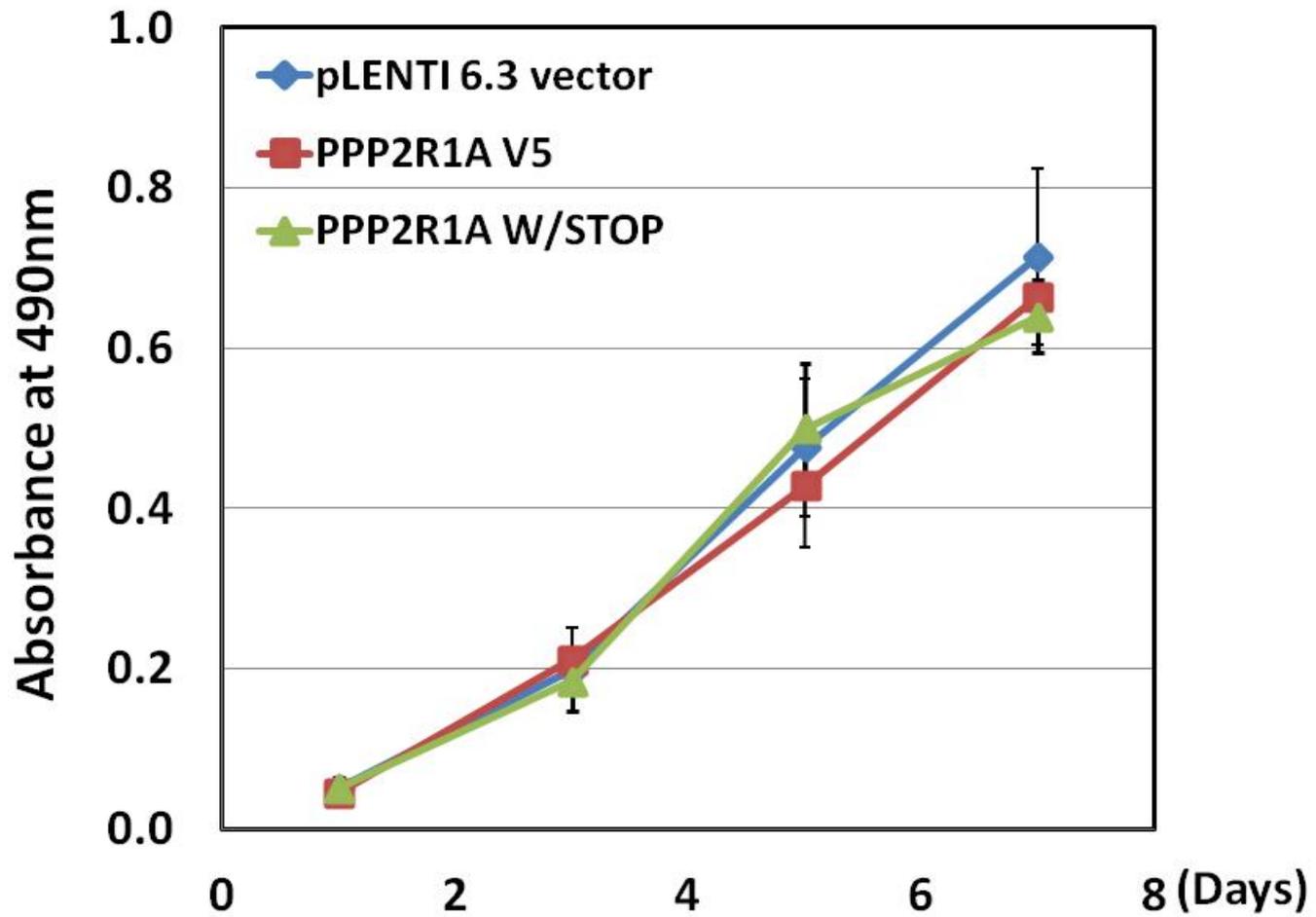
Figure 12. Proliferation assay of ACI-158 following viral introduction of PPP2R1A

Array quality check

File	Code	SF	%P+M	GAPDH 3-5-ratio	GAPDH signal
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Olga_1b_(HG-U133_Plus_2).mas5	ACI-158 V5DEST_b	2.83	49.7	1.03	32116
Olga_1c_(HG-U133_Plus_2).mas5	ACI-158 V5DEST_c	3.35	48.7	1.00	35232
Olga_2a_(HG-U133_Plus_2).mas5	ACI-158 -PPP2R1A_a	2.71	52.3	1.01	31492
Olga_2b_(HG-U133_Plus_2).mas5	ACI-158 -PPP2R1A_b	2.86	50.6	0.98	30972
Olga_2c_(HG-U133_Plus_2).mas5	ACI-158 -PPP2R1A_c	2.56	52.0	0.96	30409
Olga_3a_(HG-U133_Plus_2).mas5	EEC-hTert V5DEST_a	3.13	48.8	1.14	32699
Olga_3b_(HG-U133_Plus_2).mas5	EEC-hTert V5DEST_b	3.47	49.2	1.11	34222
Olga_3c_(HG-U133_Plus_2).mas5	EEC-hTert V5DEST_c	2.80	49.4	1.08	30551
Olga_4a_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_a	2.57	50.8	1.04	29285
Olga_4b_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_b	2.79	49.8	1.04	31833
Olga_4c_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_c	3.46	48.8	1.11	35197
Olga_5a_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut183_a	3.34	49.6	1.04	35858
Olga_5b_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut183_b	3.51	50.2	1.03	36279
Olga_5c_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut183_c	3.07	49.5	1.08	32810
Olga_6a_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut179_a	2.92	48.9	1.02	32247
Olga_6b_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut179_b	2.60	50.1	1.03	30877
Olga_6c_(HG-U133_Plus_2).mas5	EEC-hTert V5- PPP2R1A_Mut179_c	4.57	46.8	1.10	49675

Figure 13. Affymetrix human genome U133 Plus 2.0 Arrays having 54675 probe sets were used. Signal values were determined by MAS5 algorithm and total intensity normalization was applied to a target value of 500. The parameters above were estimated by Expression console software. All arrays have excellent GAPDH 3'/5' ratios. The array EEC Mut170c has higher scale factor than others.

Transcripts altered by 2-fold, at p<0.05, and a geometric average signal >100

Comparison	Up	Down	Total
ACI-158 PPP2R1A vs. V5	782	176	958
EEC-hTert PPP2R1A vs. V5	30	18	48
EEC-hTert PPP2R1A Mut183 vs. V5	42	27	69
EEC-hTert PPP2R1A Mut179 vs. V5	72	48	120
EEC-hTert PPP2R1A Mut183 vs. PPP2R1A	22	26	48
EEC-hTert PPP2R1A Mut179 vs. PPP2R1A	32	19	51

2-fold altered at p < 0.05 and signa>100
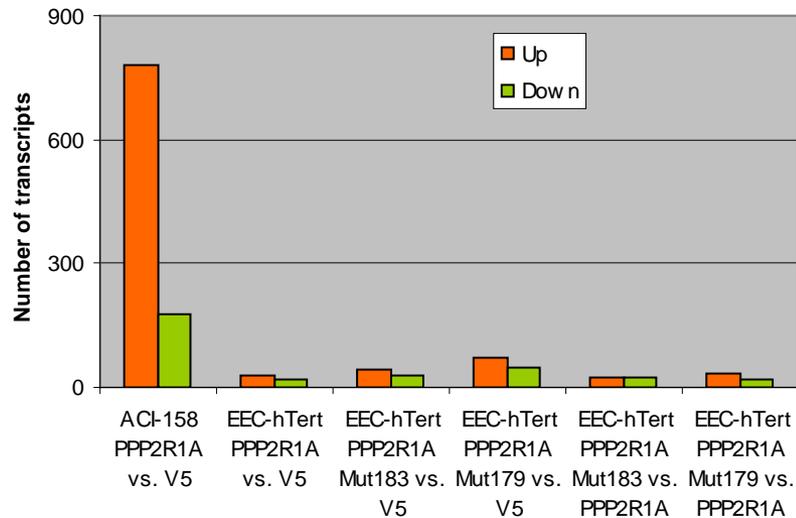
Figure 14. Differentially expressed transcripts

Figure 15 Heat map of 1165 transcripts altered by 2-fold from control at p<0.05.
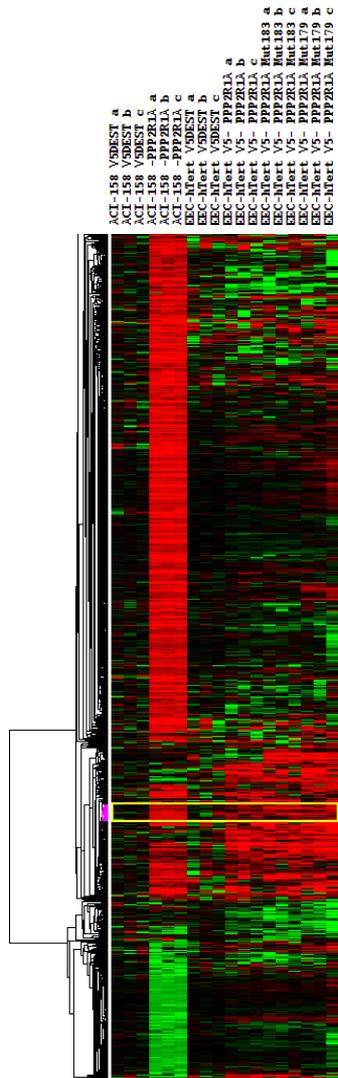
Column labels:
ACI-158 V5DEST a
ACI-158 V5DEST b
ACI-158 V5DEST c
ACI-158 -PPP2R1A a
ACI-158 -PPP2R1A b
ACI-158 -PPP2R1A c
EEC-hTert V5DEST a
EEC-hTert V5DEST b
EEC-hTert V5DEST c
EEC-hTert V5- PPP2R1A a
EEC-hTert V5- PPP2R1A b
EEC-hTert V5- PPP2R1A c
EEC-hTert V5- PPP2R1A Mut183 a
EEC-hTert V5- PPP2R1A Mut183 b
EEC-hTert V5- PPP2R1A Mut183 c
EEC-hTert V5- PPP2R1A Mut179 a
EEC-hTert V5- PPP2R1A Mut179 b
EEC-hTert V5- PPP2R1A Mut179 c
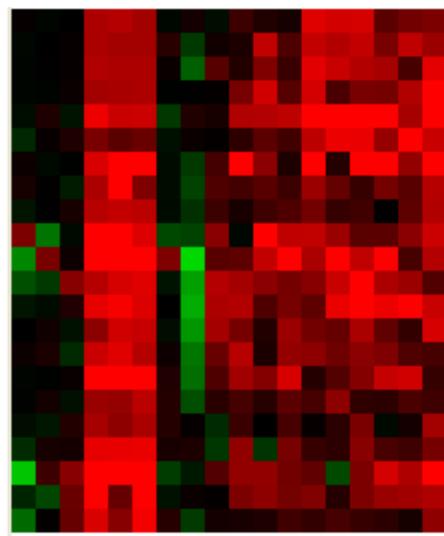
Gene list:
EGR1 - early growth response 1: 201694_s_at
SLC2A3 - solute carrier family 2 (facilitated glucose transport
EGR1 - early growth response 1: 201693_s_at
ENO2 - enolase 2 (gamma, neuronal): 201313_at
EGR1 - Early growth response 1: 227404_s_at
DUSP6 - dual specificity phosphatase 6: 208891_at
243115_at
RNF181 - Ring finger protein 181: 229932_at
FN1 - fibronectin 1: 216442_x_at
DCN - decorin: 211896_s_at
ENPP2 - ectonucleotide pyrophosphatase/phosphodiesterase 2: 209
SLAMF9 - SLAM family member 9: 1553770_a_at
RND1 - Rho family GTPase 1: 210056_at
CES3 - carboxylesterase 3: 220335_x_at
ACRC - acidic repeat containing: 238825_at
IFI44 - interferon-induced protein 44: 214453_s_at
MTMR11 - myotubularin related protein 11: 205076_s_at
PRKACB - protein kinase, cAMP-dependent, catalytic, beta: 23578
SAMD9L - sterile alpha motif domain containing 9-like: 226603_a
LOC728431 - Hypothetical LOC728431: 229678_at
VWA1 - von Willebrand factor A domain containing 1: 218731_s_at
239991_at

<1/3   1   >3

Ratio to control V5
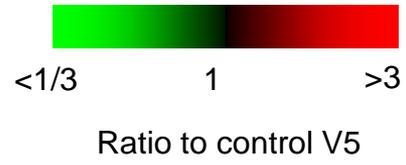
Figure 16. Multidimensional scaling plot of *z-scores* of about 24K transcripts having at least 1 present call and differentially expressed at p < 0.1 in any one comparison with control V5 or WT PPP2R1A.

z-scores were separately calculated for each cell line in order to equalize variance between the two cell lines.
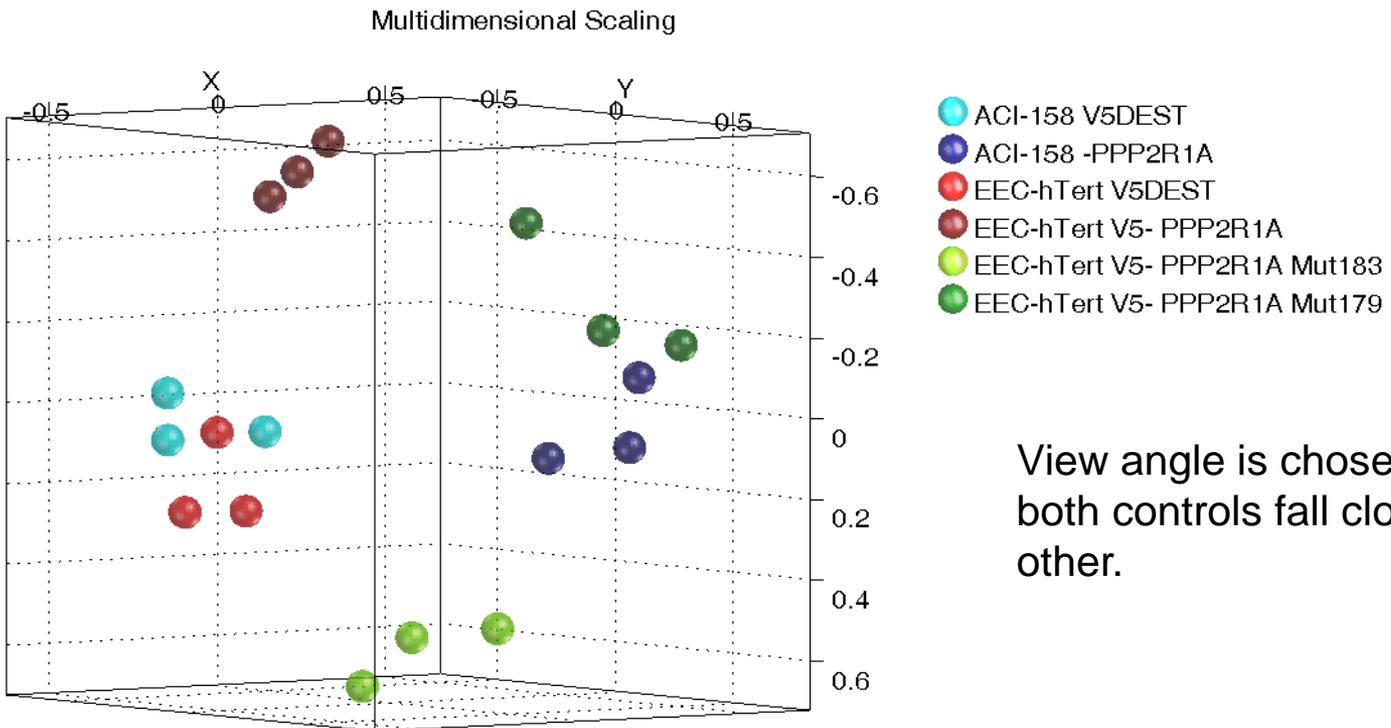
View angle is chosen such that both controls fall close to each other.
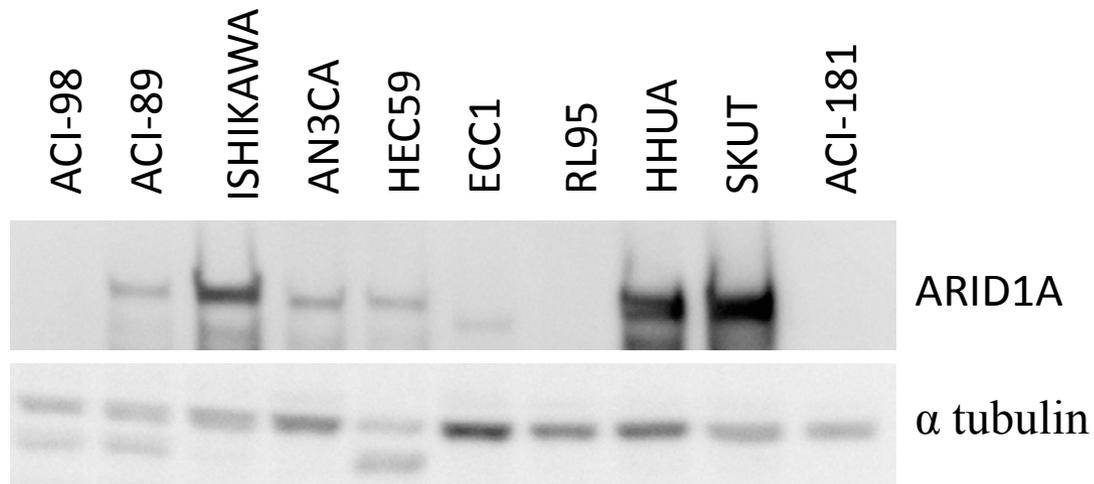
Figure 17. **ARID1A expression**. Immunoblot of ARID1A in cell extracts of ovarian and uterine cancer cell lines. Tubulin serves as loading control. 3-8% SDS PAGE
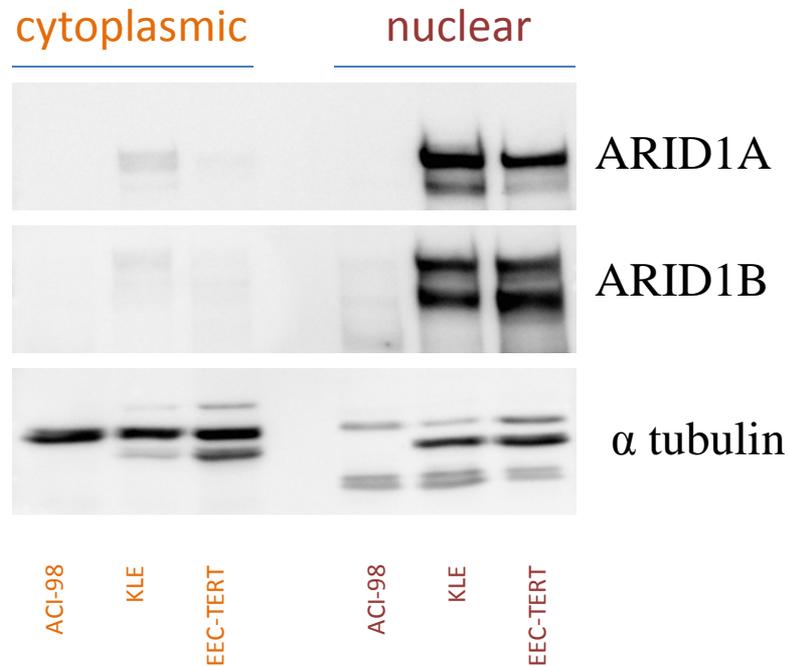
Figure 18 . Immunoblots of nuclear and cytoplasmic cell extracts of endometrial cell lines. KLE endometrial cancer and normal EEC-TERT express nuclear expressed ARID1A and ARID1B wheras ACI-98 cells do not express either protein

ARID1A

1 2 3 4 5 6 7

268
238

ARID1B

7 6 5 4 3 2 1

268
238
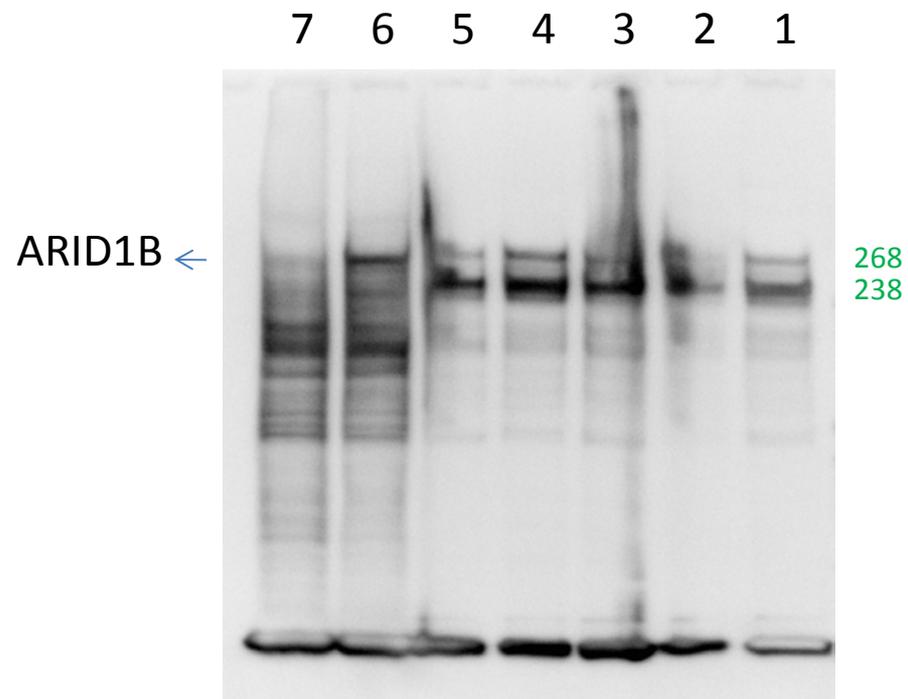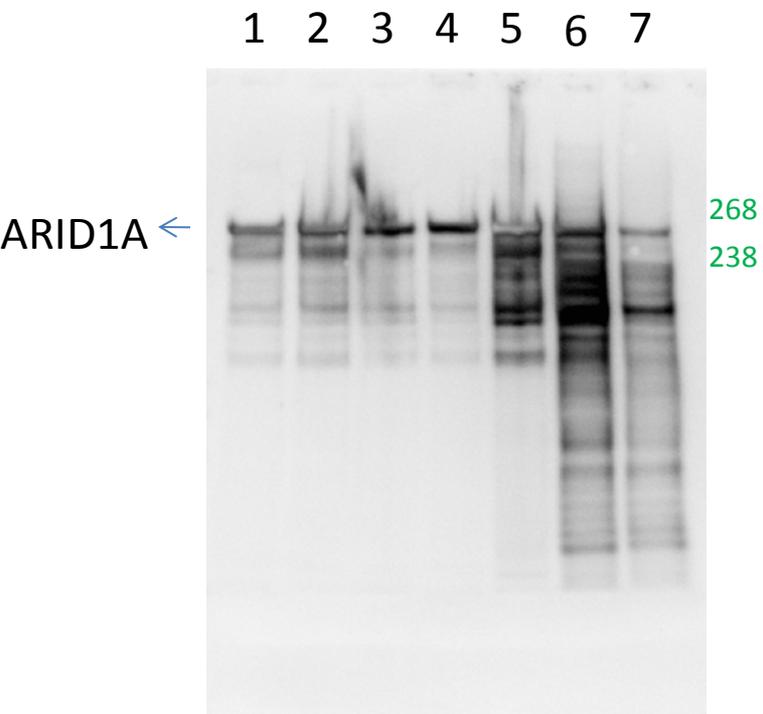
Figure 19. Immunoblots of DES
induced murine endometrial
carcinoma cell lines for Arid1A
and Arid1B expression. Lanes 4
and 5 murine positive control
and 6 and 7 human lines control

1 - DES 092788-1
2 – DES 0921881
3 – DES 0914881
4 – NIH 3T3
5 – MMT060562
6 – KLE
7 – ACI-158
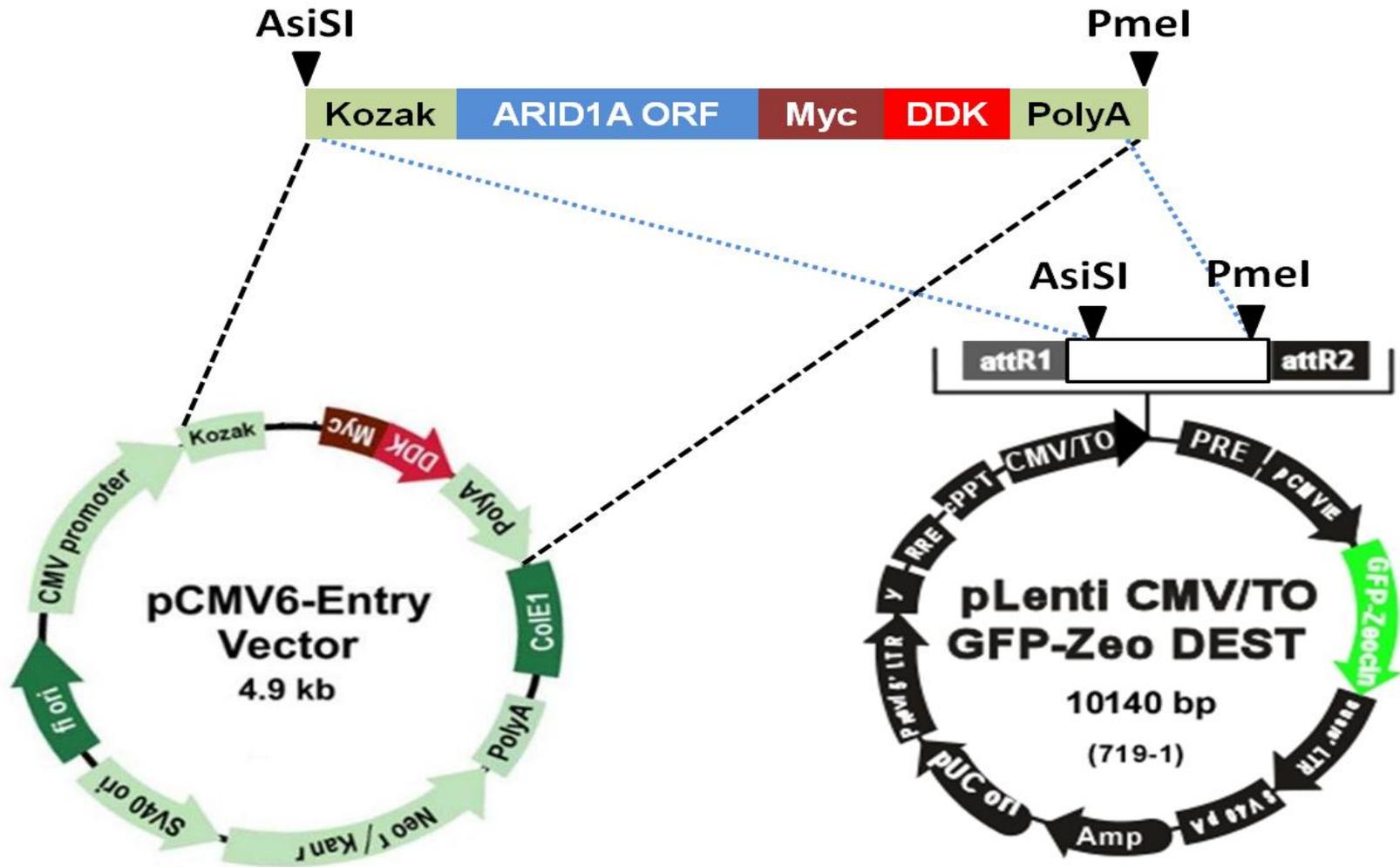
Figure 20. schematic of ARID1A cloning and Tet/ON vector re-engineering

ARID1B plasmid construct

ORF of ARID1B / Myc-Flag tag
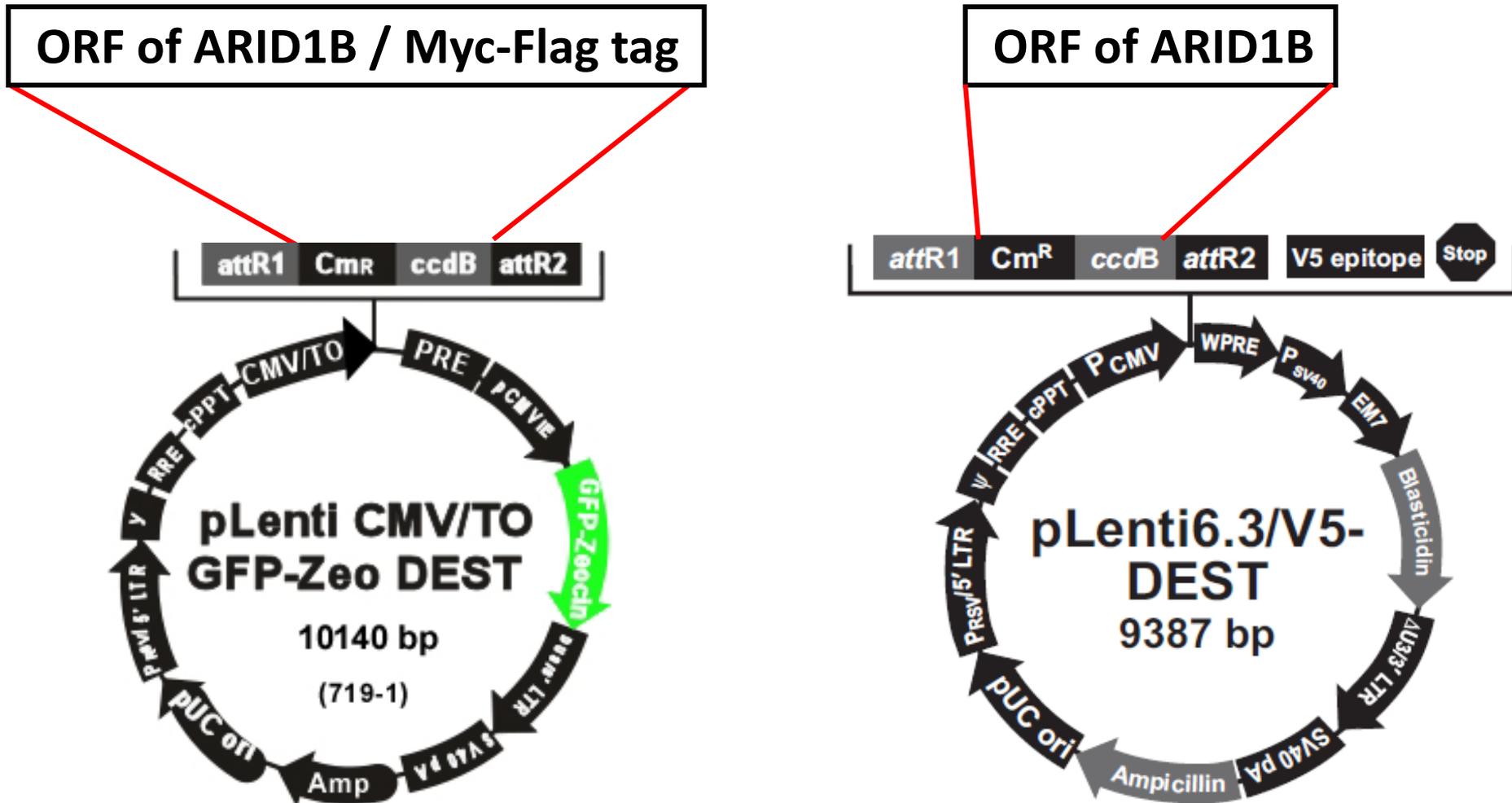
ORF of ARID1B

Figure 21. Schematic of ARID1B cloning and conventional Lenti and Tet/ON lenti vector

Transfection of pLenti CMV_TO ARID1A to 293FT

Bright field

GFP

pLenti CMV_TO vector   pLenti CMV_TO ARID1A
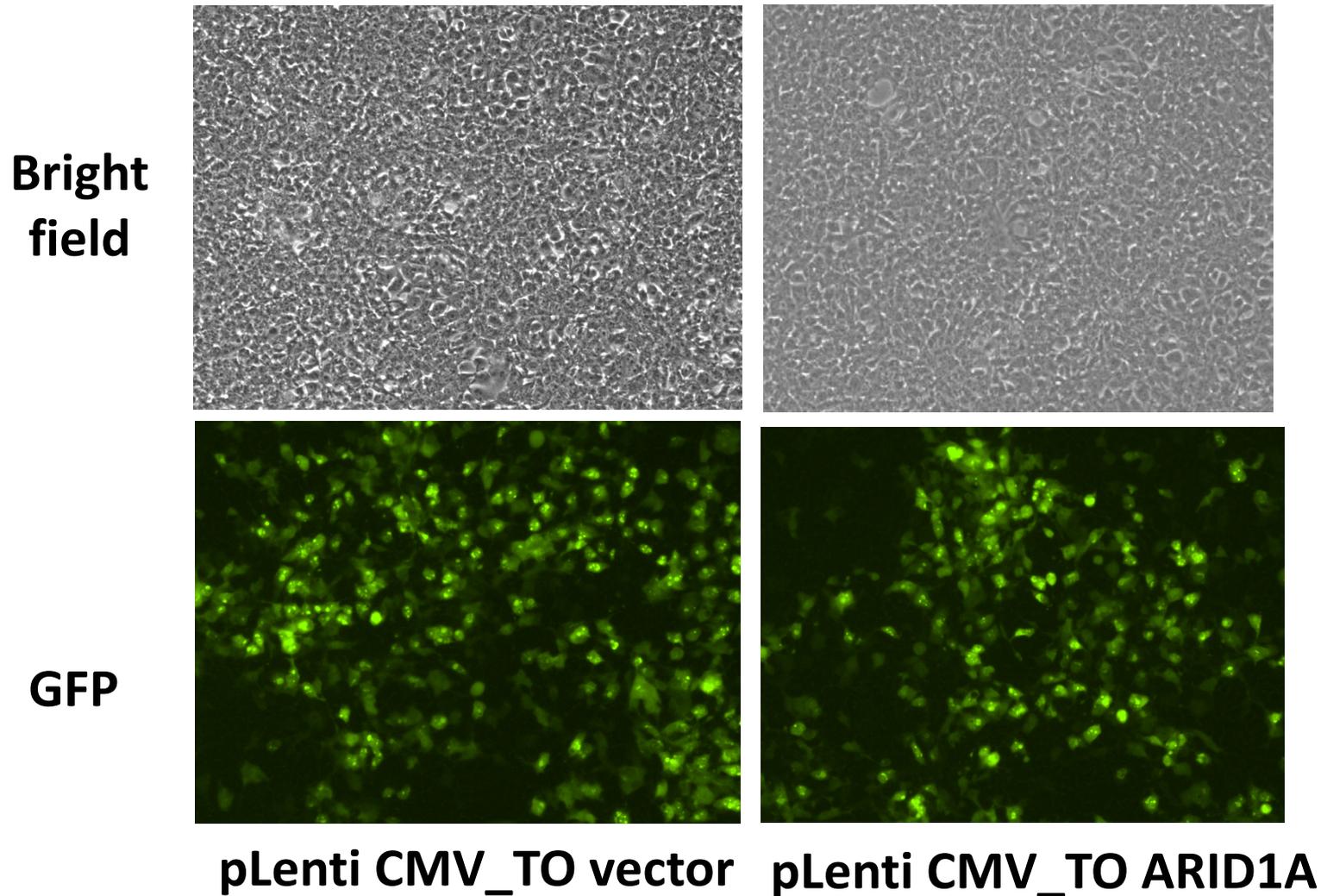
Figure 22. Initial ARID1A vector transfection control. Efficiency monitored by GFP immunoflourescence

WB of recombinant ARID1A
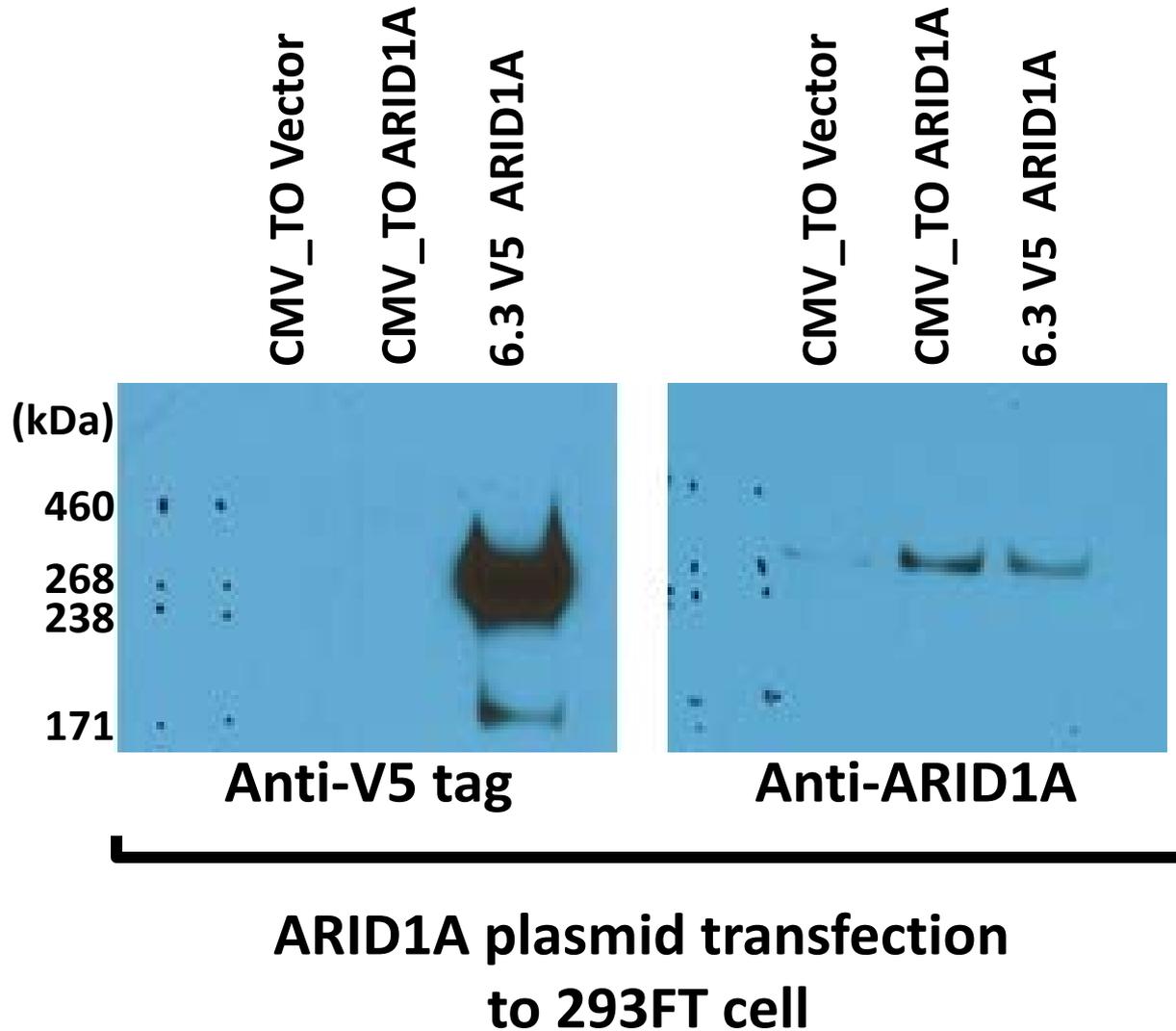
Figure 23. Immunoblots demonstrating recombinant ARID1A in 293 cells.

Production of ARID1A Lentivaral

Co-transfect

293FT cell

Lentiviral packaging plasmid

WB of recombinant ARID1A

ARID1A plasmid transfection to 293FT cell

Anti-V5 tag

Anti-ARID1A

ARID1A lentivirus transduction to 293FT cell

Anti-V5 tag

CMV_TO Vector
CMV_TO ARID1A
6.3 V5 ARID1A

(kDa)
460
268
238
171

Establish of TetR expression cell

pcDNA6/TR© 6662 bp

pLenti CMV/TO GFP-Zeo DEST 10140 bp (719-1)

attR1 CmR ccdB attR2
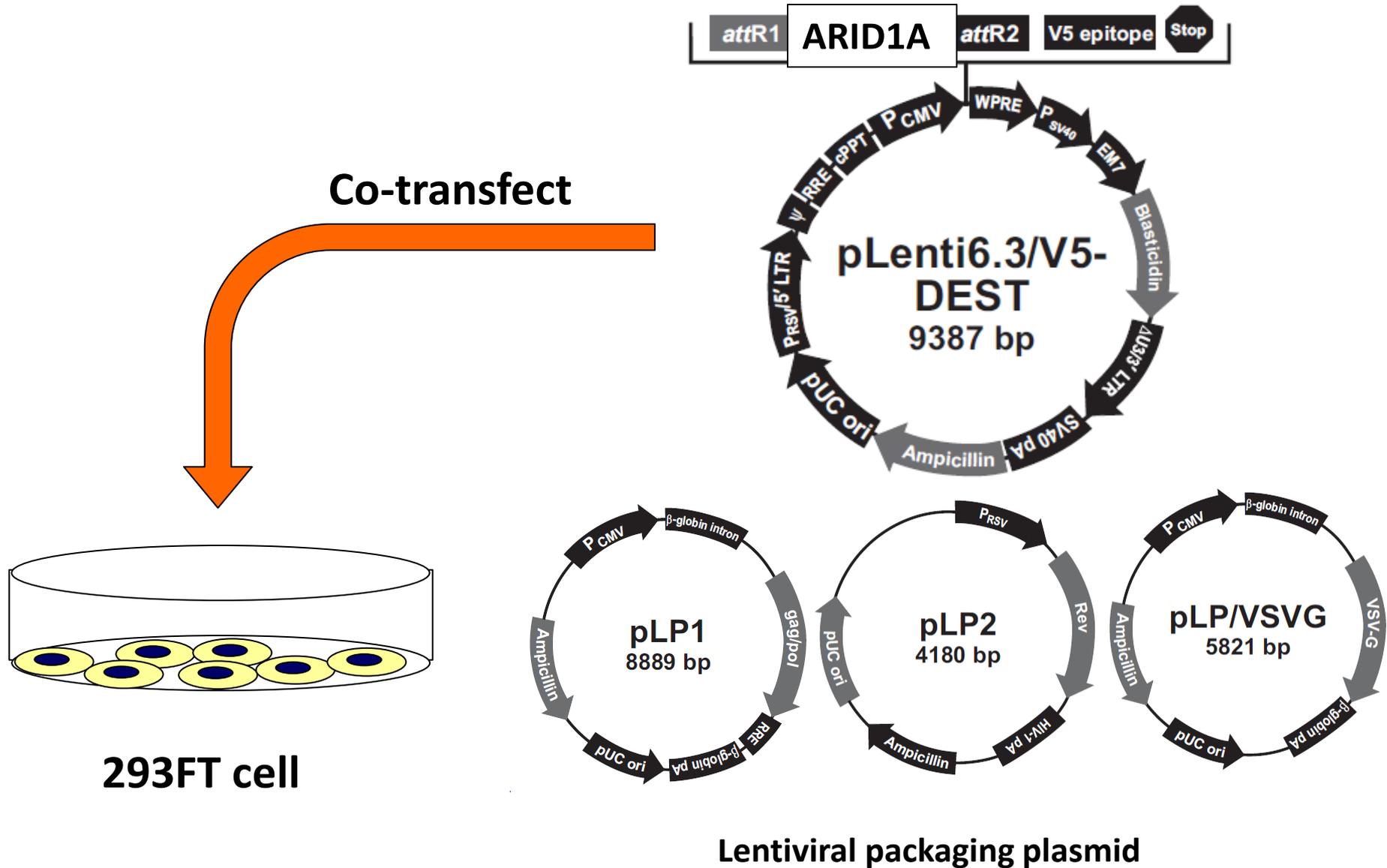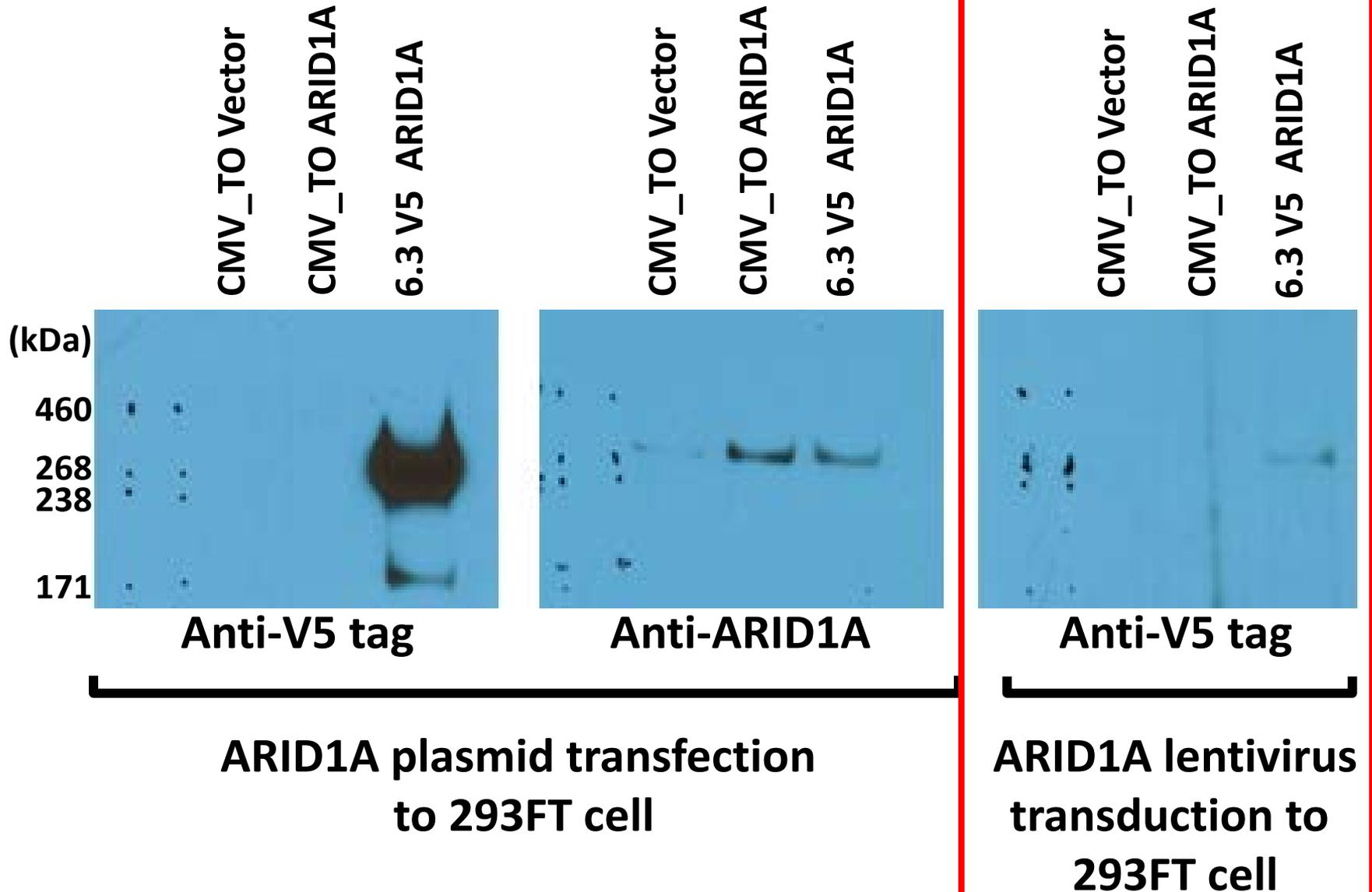
ARID1A lentivirus
(Zeocin resistance)
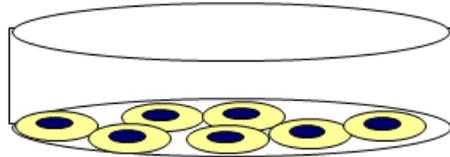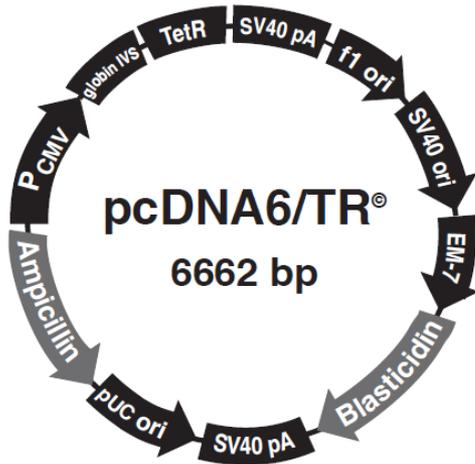
TetR expression cell
(Blasticidin resistance)
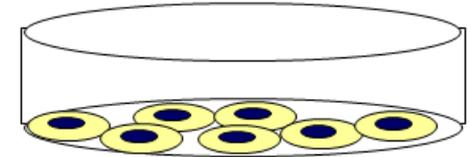
inducible ARID1A

❖ **Production of high-titer virus and/or high transduction efficiency**

❖ **Establish of TetR expression cell**
   (Ovarian clear cell carcinoma cell line TOV21G and ACI-89, ARID1A negative)

❖ **ARID 1B plasmid construction**

Target region of ARID1A shRNA lentivirus from Sigma

NM_006015 ARID1A Variant 1 (ORF 6858bp)

#10 exon2-3   #3 exon3   #9 exon18   #1, #2 exon20

NM_139135 ARID1A Variant 2 (ORF 6207bp)

#10, Sigam TRCN0000059090 : exon 2-3 (Guan B., et al, Cancer Res. 2011)
#3, Sigma TRCN0000344652 : exon3
#9, Sigma TRCN0000344709 : exon 18 (specific to variant1)
#1, Sigam TRCN0000059091 : exon 20 (Guan B., et al, Cancer Res. 2011)
#2, Sigma TRCN0000333242 : exon 20

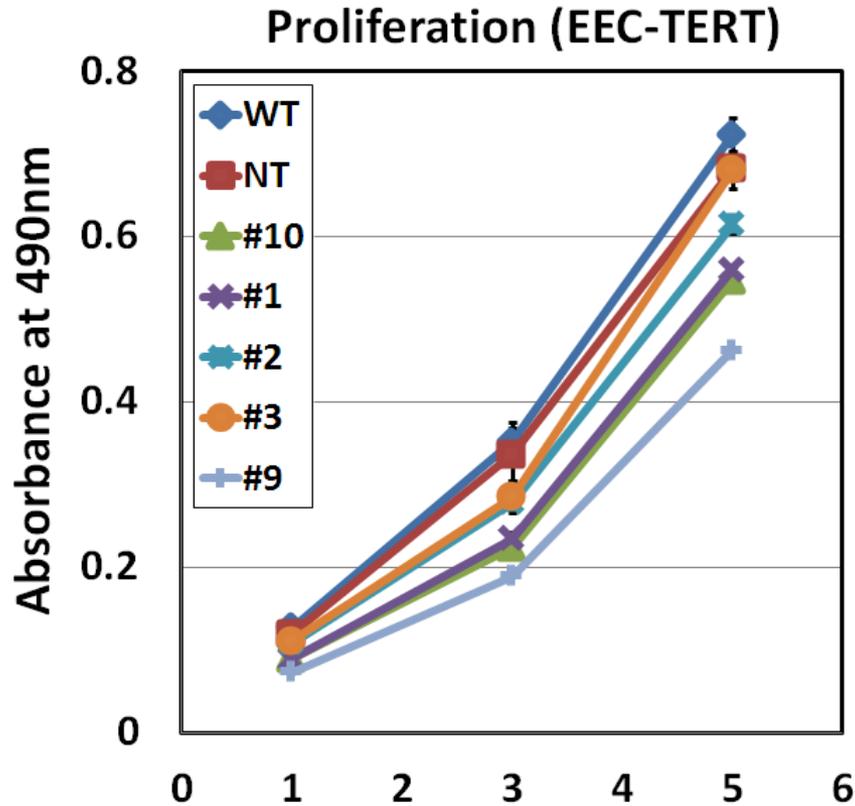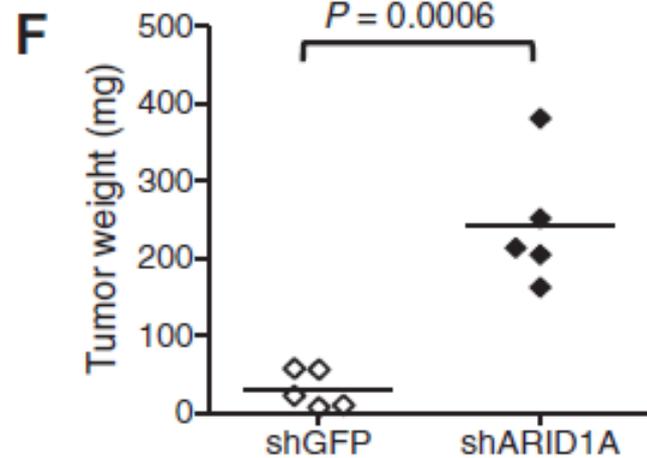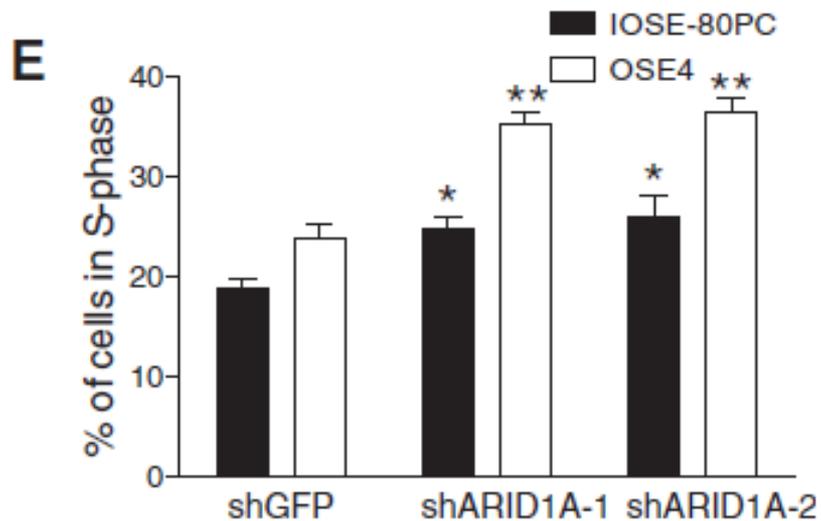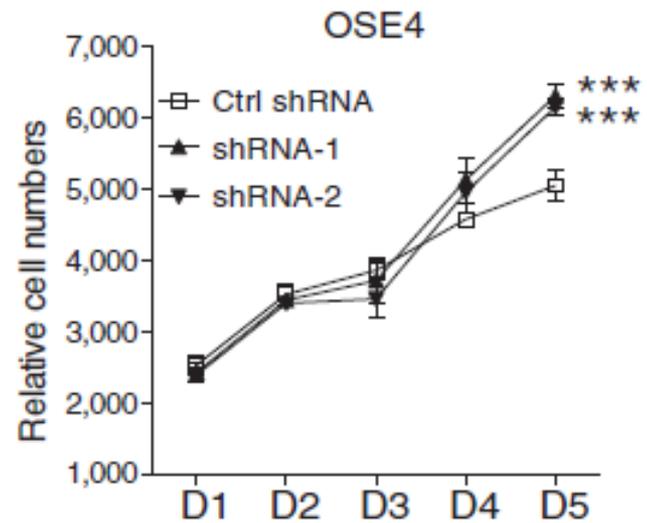# Knockdown efficiency of shARID1A (EEC-TERT)
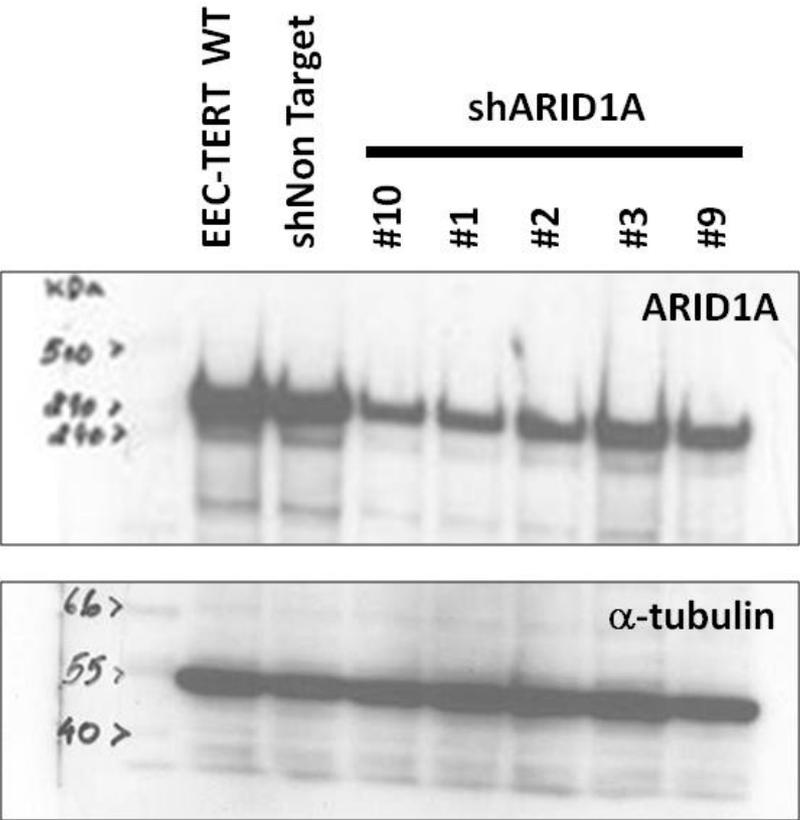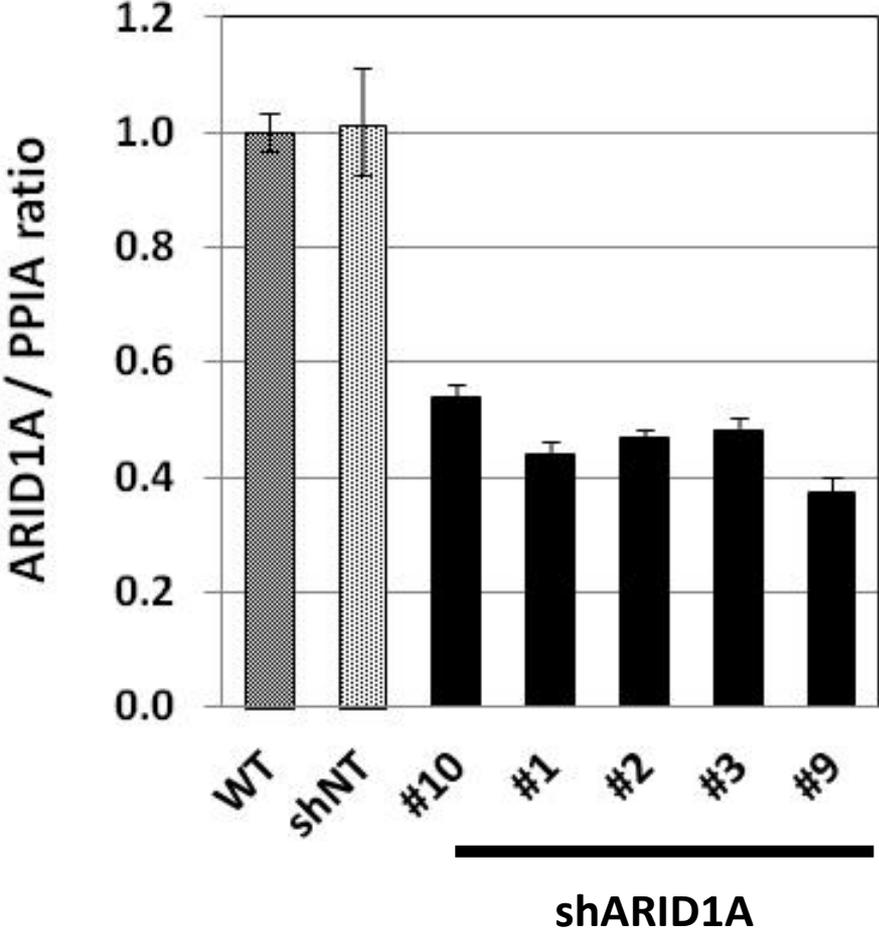
Proliferation of shARID1A / EEC-TERT
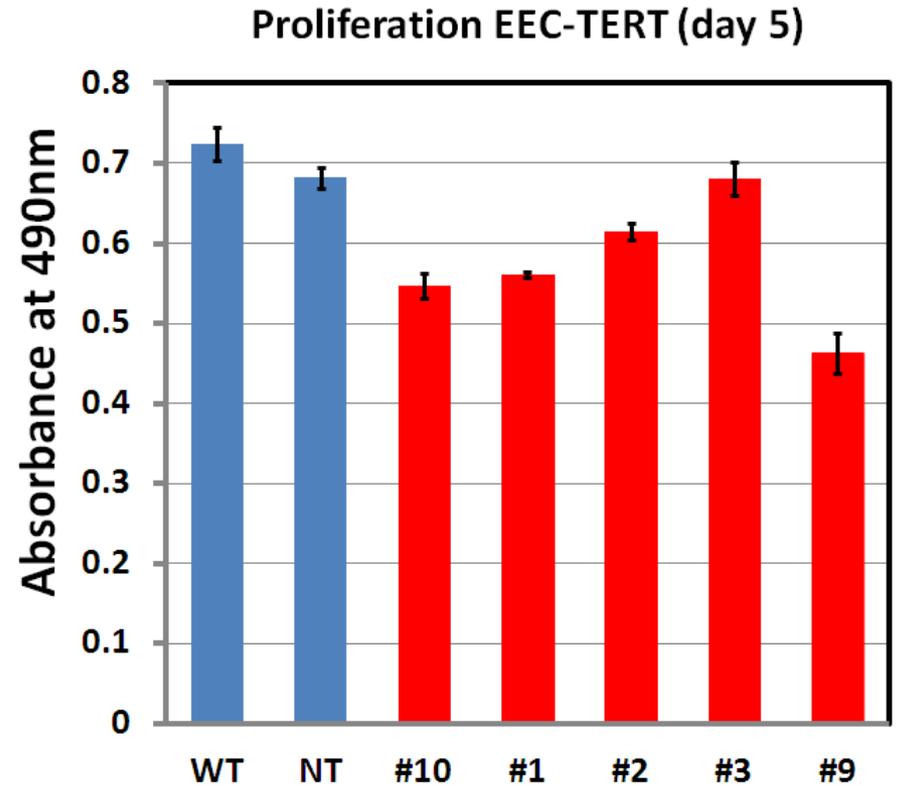
On going : H118 & HIO-103 (ovarian surface epithelial cells)

D

IOSE-80PC

- □ Ctrl shRNA
- ▲ shRNA-1
- ▼ shRNA-2

***
***

OSE4

- □ Ctrl shRNA
- ▲ shRNA-1
- ▼ shRNA-2

***
***

E

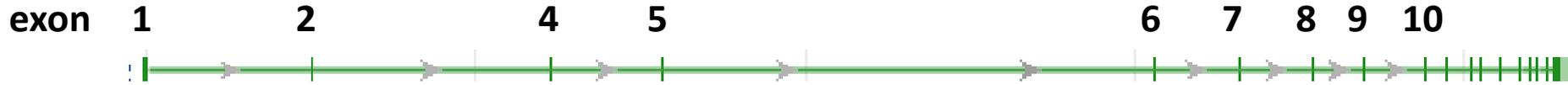- ■ IOSE-80PC
- □ OSE4

F

$P = 0.0006$
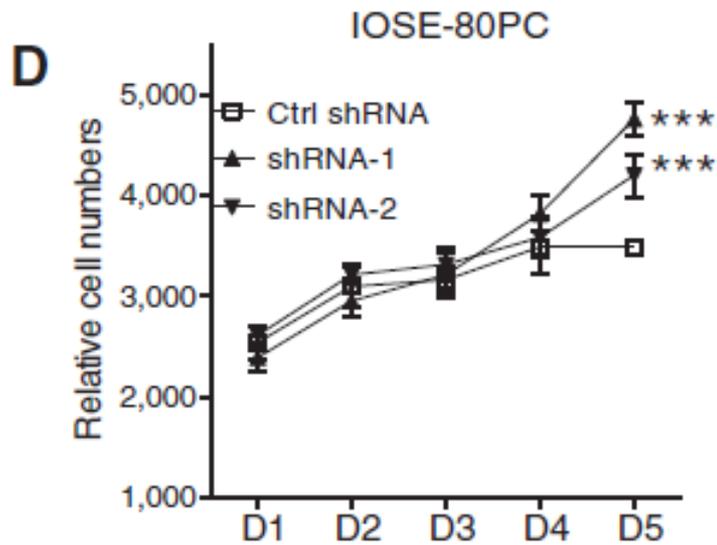
OSE4, IOSE-80PC : Ovarian suface epithelium (immortalized by SV40 TAg)
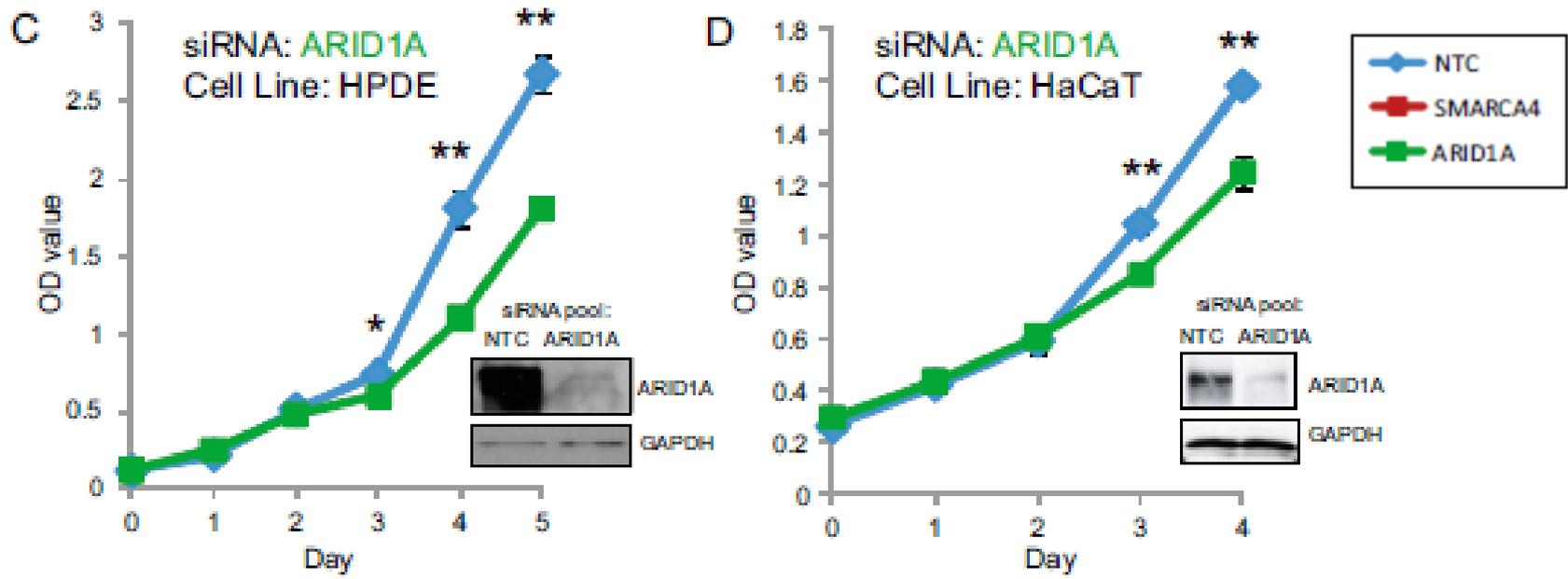
HPDE : Human pancreatic duct epithelial (immortalized by SV40 TAg)
HaCaT : Human skin keratinocyte (spontaneously transformed)

Shain AH., et al. PNAS. 2012

Target region of ARID1B shRNA lentivirus from Sigma
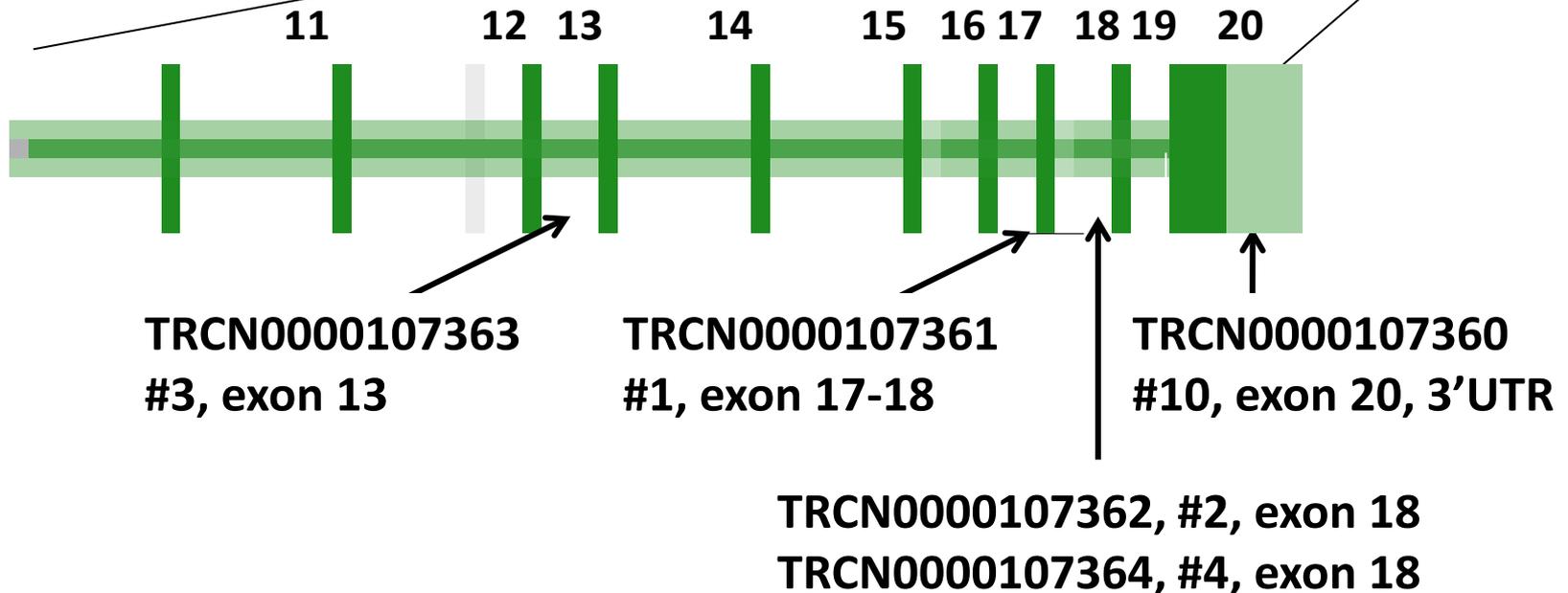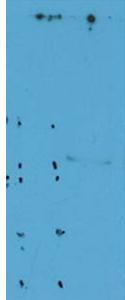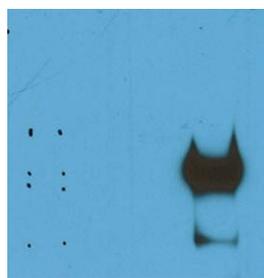
Tet-On System
(Transcription turned on by addition of Dox)

Integrated copy of pTet-On regulator plasmid

− Dox

+ Dox

rTetR

rtTA

rTetR

TRE P_{minCMV}

Transcription

Integrated copy of pTRE response plasmid
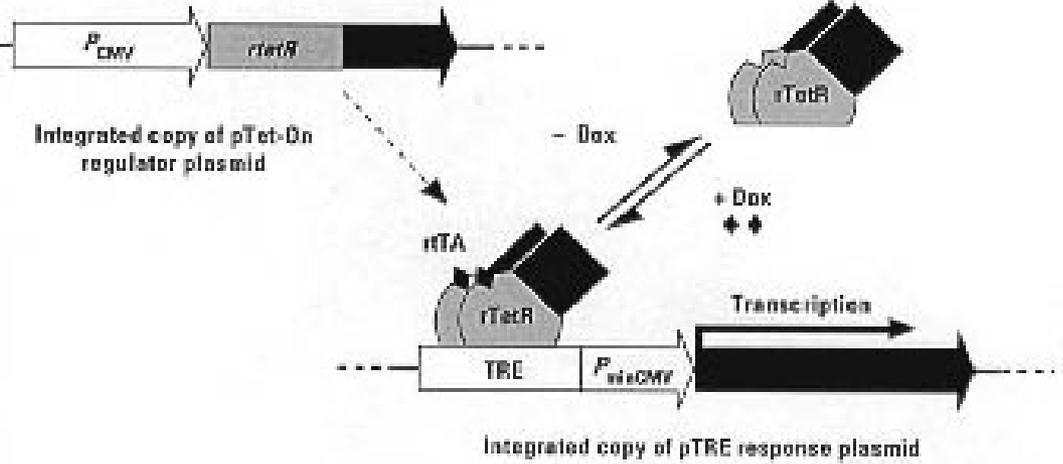
1.   Hombach-Klonisch S, Kehlen A, Fowler PA, Huppertz B, Jugert JF, Bischoff G, et al. Regulation of functional steroid receptors and ligand-induced responses in telomerase-immortalized human endometrial epithelial cells. J Mol Endocrinol. 2005;34(2):517-34.
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3.   Shih Ie M, Panuganti PK, Kuo KT, Mao TL, Kuhn E, Jones S, et al. Somatic mutations of PPP2R1A in ovarian and uterine carcinomas. Am J Pathol.178(4):1442-7. PMCID: 3078449.
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5.   Jones S, Wang TL, Shih Ie M, Mao TL, Nakayama K, Roden R, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science.330(6001):228-31. PMCID: 3076894.
6.   Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med.363(16):1532-43. PMCID: 2976679.
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10.   Sherman ME, Devesa SS. Analysis of racial differences in incidence, survival, and mortality for malignant tumors of the uterine corpus. Cancer. 2003;98(1):176-86.
11.   Hicks ML, Phillips JL, Parham G, Andrews N, Jones WB, Shingleton HM, et al. The National Cancer Data Base report on endometrial carcinoma in African-American women. Cancer. 1998;83(12):2629-37.
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15.   Santin AD, Bellone S, Siegel ER, Palmieri M, Thomas M, Cannon MJ, et al. Racial differences in the overexpression of epidermal growth factor type II receptor (HER2/neu): a major prognostic indicator in uterine serous papillary cancer. Am J Obstet Gynecol. 2005;192(3):813-8.
16.   Morrison C, Miecznikowski J, Darcy KM, Dolce JM, Kandel E, Erwin DO, et al. A GOG 210 aCGH study of gain at 1q23 in endometrioid endometrial cancer in the context of racial disparity and outcome. Genes Chromosomes Cancer.49(9):791-802.
17.   Powell MA, Mutch DG, Rader JS, Herzog TJ, Huang TH, Goodfellow PJ. Ribosomal DNA methylation in patients with endometrial carcinoma: an independent prognostic marker. Cancer. 2002;94(11):2941-52.

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