Assessing estrogen-induced proliferative response in an endometrial cancer cell line using a universally applicable methodological guide.

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Abbreviations: Endometrial Cancer (EC), Ishikawa (ISK), Estrogen Receptor (ER), Androgen Receptor (AR), Progesterone Receptor (PR), 17 β-estradiol (E2), Polyacrylamide gel electrophoresis (PAGE), Short Tandem Repeat (STR), Charcoal stripped fetal bovine serum (CSFBS), Chorioallantoic Membrane (CAM), Neutral Buffered Formalin (NBF), Phosphate Buffered Saline (PBS), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ), Peptidylprolyl Isomerase A (PPIA)

Abstract

Objective: Translational endometrial cancer (EC) research benefits from an *in vitro* experimental approach using EC cell lines. We demonstrated the steps that are required to examine estrogen induced proliferative response, a simple yet important research question pertinent to EC and devised a pragmatic methodological workflow for utilising EC cell lines in experimental models.

Methods/materials: Comprehensive review of all commercially available EC cell lines was carried out, and Ishikawa cell line was selected to study the estrogen responsiveness with HEC1A, RL95-2 and MFE280 cell lines as comparators where appropriate, examining relevant differential molecular (steroid receptors) and functional (phenotype, anchorage-independent growth, hormone responsiveness, migration, invasion and chemosensitivity) characteristics in 2D and 3D cultures *in vitro* using immunocytochemistry, immunofluorescence, qPCR and western blotting. *In vivo* tumour, formation and chemosensitivity were also assessed in a chick chorioallantoic membrane (CAM) model.

Results: Short Tandem Repeat (STR) analysis authenticated the purchased cell lines while gifted cells deviated significantly from the published profile. We demonstrate the importance of prior assessment of the suitability of each cell line for the chosen in vitro experimental technique. Prior establishment of baseline, non-enriched conditions was required to induce a proliferative response to estrogen. The CAM model was a suitable *in vivo* multi-cellular animal model for EC, for producing rapid and reproducible data.

Conclusions: We have developed a methodological guide for EC researchers when using endometrial cell lines to answer important translational research questions

(exemplified by estrogen responsive cell proliferation), to facilitate robust data, while saving time and resources.

Keywords : Endometrial cancer, cell lines, estrogen

Introduction

Endometrial cancer (EC) is the fourth commonest malignancy in women in Europe (6% of all female cancers) [1, 2]. The increasing rates of obesity are projected to increase EC incidence by up to 100% by 2025, relative to the observed incidence in 2005 in some European countries [3]. EC-associated mortality has increased by 15% with many patients unsuitable for standard surgical treatment due to co-morbidities and 1 in 4 women experiencing serious surgical complications [4-6]. Current therapeutics also fail to treat late stage disease and, similar to ovarian cancer, survival rates are especially poor for advanced EC [7]. Despite all of the above, compared with other hormonally driven malignancies such as breast or ovarian cancer, EC is a relatively under-researched area. Further research, therefore, is urgently needed to formulate effective preventative and treatment modalities.

The advancement of cancer therapy is dependent on the understanding of the pathogenesis associated molecular biology, which in turn is reliant on the availability of model systems. For basic science research, cell lines offer a relatively cheap and high-throughput model for screening potential biomarkers and therapeutic targets in a relatively quick and reproducible manner. Over 80% of ECs are carcinomas, and the first EC cell line HEC-1 was established in 1968 [8].

The cell line that best represents the *in vivo* physiology and pathology of ECs can be determined by employing many phenotypic and functional characteristics. Due to the nature of continuous culture, potential contamination and spontaneous mutations can occur in these cell lines [9], representing a major potential confounding factor affecting experimental outcomes. The aim of this paper is to demonstrate how a pertinent

research question in EC cancer can be answered in vitro using an endometrial cell line, with a selective methodological process that we developed.

Using the established epithelial EC cell line, Ishikawa (ISK), and three other established, commercially available and commonly used EC cell lines as required, we illustrated how the important EC function, estrogen induced cellular proliferative response could be a model in vitro. In doing so we developed a methodological guide that can be employed to model some known features of EC, (1) estrogen induce proliferation, characterising the differential molecular phenotype (hormone receptor expression (ERα, ERβ, AR, PR)) and functional properties (gene expression and proliferation) *in vitro*; and (2) chemosensitivity, illustrating the importance of initially establishing the basic functional features of the cell line (anchorage-independent growth, migration, and invasion *in vitro* and tumour formation *in vivo* in CAM). Our methodological guide will aid researchers considering using endometrial cell lines for their research.

Materials and Methods

Extended methods can be found in the supplementary methods section.

Cell lines:

Four established, endometrial cell lines; ISK, HEC1A [10] and RL95-2 [11] [12] and MFE280 [13] were selected and sourced from reputable commercial biobanks (ATCC and Public Health England (PHE)); which routinely screen their cell lines for contamination and genetic abnormalities. Furthermore, a highly passaged HEC1A cell line which was sourced from a collaborator was also used.

STR Profile Analysis

DNA was extracted using the Wizard SV Kit (Promega, Southampton, UK) according to the manufacturer's protocol and sequenced using Promega Powerplex® 16 System, for tissue culture strain identification. Profile results were compared with published STR profiles for each cell line from ATCC, PHE Culture collections.

Cell Culture

All reagents were supplied by Sigma, Dorset, UK unless otherwise stated, cell lines were maintained in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS, Biosera, UK), L-glutamine and penicillin/streptomycin benzyl penicillin at 37ºC in a 5% (v/v) CO_2 atmosphere and details of cell line maintenance and modifications relevant to different assays are detailed in the supplementary methods section.

Immunostaining

Immunocytochemistry: Cells were grown in 8-well glass slide chambers until at least 60% confluent, washed with PBS and fixed with 10% (v/v) Neutral Buffered Formalin (NBF) and permeabilized with 0.2% (v/v) Triton X-100 (BD Biosciences, Oxford, UK) in PBS. 3 µm-thick paraffin sections of tumour samples grown on CAM were prepared for immunostaining as previously described [14].

Immunofluorescence: Unspecific antigens were blocked with 2.5% normal horse serum and incubated with primary antibodies overnight at 4ºC. Cells were washed and incubated with the secondary antibodies for 1h in the dark (antibodies used detailed in Supplementary methods, and (Supplementary Table.. S6). Samples were then mounted (Vectorshield and Dapi mounting media (Vector Labs)) and visualised with an Eclipse 50i microscope (Nikon) using a mercury lamp. NIS-Elements F software

and ImageJ were used for image capture. For confocal images, fast Nipkow disc-based confocal imaging attached to a high sensitivity (iXon Andor) CCD camera was used at the same intensity and compared with IgG controls.

RNA Extraction and Real Time-qPCR

RNA was extracted, quantified and reverse transcribed according to previously described method [15]. cDNA was amplified using iTaq Universal SYBR Green Supermix and the CFX Connect Real-Time System (Bio-Rad, Hercules, CA). Primers and reaction conditions are listed in Supplementary Table S5. Relative transcript expression was calculated by the ΔΔCT method, normalised to the reference genes Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ) as previously described [15] and Peptidylprolyl Isomerase A PPIA [16] using Bio-Rad CFX Manager (Bio-Rad, Hertfordshire). Modified ERβ cDNA amplification step detailed in supplementary methods.

Protein extraction and SDS-PAGE

Protein lysates were prepared, quantified and analysed by SDS-PAGE as described previously [15] for the phosphorylated antibodies. For total ERα blots, samples were sonicated, and lysates were diluted in x5 Lammeli buffer, electrophoresed through 10% (w/v) polyacrylamide gels. Primary antibodies used were phospho-estrogen receptor α (ERα antibody sampler kit (#9024, Cell Signalling, MA), anti-ERα (Abcam) validated against tubulin (Sigma: for ERα) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH). by densitometry using ImageJ [17]

Assay for Cell Viability and Proliferation

Cell viability and proliferation was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [18]. Cell viability was

expressed as a percentage of the untreated control. Experiments were run in triplicate with eight technical replicate wells.

Scratch Migration Assay

Linear scratch was made through each confluent monolayer (after overnight attachment) in 6- well plates, and perpendicular guidelines were drawn on the underside of the plate intersecting the scratch at 3 points per well. The wells were washed with PBS after the scratch, to remove any suspended cells, then medium was gently reapplied. Images were taken at each guideline at 0h, 4h, 6h, 24h and 48h and analysed using MiToBio software plugin [19] for ImageJ [17].

Chorioallantoic Membrane Model

ISK, and HEC1A cells were labelled with an EGFP-expressing lentivirus donated by Dr Sokratis Theocharatos (University of Liverpool, UK). RL95-2 cells were labelled with a lentivirus expressing tGFP (pGFP-C-shLenti plasmid) (Origene, MD, USA), and selected using puromycin antibiotic selection. ISK and HEC1A viral transfection and egg preparation were performed according to Carter et al. 2012 [20]. Cells were added to the CAM at embryonic day 7 (E7) [21] the resulting tumours were imaged at E14, using a standard Leica M165-FC fluorescent microscope, *in situ* and after excision. (Fig. 5C) the resulting tumours were fixed in 10% (v/v) NBF and embedded in paraffin wax.

Statistical Analysis

Statistical analyses was performed using GraphPad Prism software using independent sample t-test to determine the difference between hormone regulation and transwell migration experiments. The criterion for significance was $p \leq 0.05$. Data

are presented as mean ± standard error of the mean (SEM). MTT, scratch and FACS analysis analysed using independent samples t-test.

Results

Selection of the appropriate cell line(s)

Figure S1 illustrates all currently available endometrioid EC cell lines that are available for researchers worldwide from commercial suppliers. We focussed on hormone responsive endometroid (or type 1) EC, to establish an in vitro model to examine estrogen induced proliferative response. Therefore, we selected the ISK cell line, which is known to express the full complement of steroid receptors, and three further cell lines (HEC1A, MFE280, and RL95-2) that originated from endometrioid tumours and exhibit a range of differentiation and hormone receptor expression, as comparators to ISK to illustrate different experimental approaches with alternative cell lines. Cell line details are in Supplementary Fig. S7.

Authenticating cell lines

As expected, the STR profile of the 4 commercially obtained cell lines exhibited their published profile (Supplementary Table. S2) [22] initially and at the end of our experimental process only with a few minor peaks indicating the beginnings of genetic drift but well within the 80-100% profile match (example in Fig. 1). However, the gifted HEC1A cell line (sourced 25 years ago, estimated to be passaged for more than 30 times) deviated significantly from the published profile, with the omission and addition of multiple peaks, and cannot therefore be reliably used as HEC1A cells for comparison to other studies using the validated HEC1A cells (Fig. 1A).

Characterisation of the cell lines;

Morphology

The 4 selected cell lines were characteristically epithelioid in 2D culture (Fig. 1B), although the RL95-2 cell line exhibited a strong tendency to pile up in gland-like dome structures [23] with time in culture rather than form confluent monolayers like Ishikawa and HEC1A. The MFE 280 cell line tended to form discrete islands of cell clusters and presumably reflects that they were originally grown as aggregates in suspension culture [13]. In 3D culture with Matrigel, ISK (Fig. 1C), HEC1A and RL95-2 produced spheroids (Fig. 5B). There was no evidence of lumen formation under the culture conditions (Fig. 1C IV).

Designing the experiment

ISK cells expressing the full complement of steroid receptors was selected for testing our primary focus, modelling estrogen induced cell proliferation in EC. Comparisons were made between ISK and RL95-2 cells for most of the subsequent experiments considering the growth and the steroid hormone expression pattern. More invasive HEC1A cells (yet with a similar 2D growth pattern) were also examined in the migration and invasion experiments.

Target Modulation

Estrogen is the main driver of EC, and through modifying the expression of the other steroid receptors it modulates the general steroid responsiveness in EC cells, thus was our molecular target; therefore herein we demonstrate the characterization of ER expression (Fig. 2 & Supplementary Fig. S3) and the subsequent pharmacological manipulation of ER using the main naturally occurring ligand (E2) in the ISK cell line.

Hormone Regulation

Estrogen is known to drive EC cell proliferation through ERα, therefore ERα mRNA and protein expression was confirmed in the ISK cell line (Fig.2-A-2C). ERα mRNA (ESR1) and protein were also expressed by MFE280 while low/undetectable mRNA and protein levels were observed in RL95-2 and HEC1A (Fig. 2A & 2C). All four cell lines retained detectable mRNA and protein for the ERβ subtype (Fig.1A). The expression of other hormone receptor gene/protein was also examined, demonstrating that the ISK cell line express mRNA and protein for all four steroid receptors (ERα, ERβ, AR and PR) compared with the other three cell lines (Fig. 2A - 2D).

In ISK cells, the concentration of E2 and charcoal stripped FBS (CSFBS) were the main variables determining the proliferative response to estrogen. E2 increased ERα protein and it was phosphorylated on multiple serine residues (Fig. 2F & Supplementary Fig.S3A) in response to a range of E2 concentrations after 72h treatment (10^{-12}-10^{-6}M; Supplementary Fig. S3). In response to E2 binding, human ERα is predominately phosphorylated on Ser-118 and to a lesser extent on Ser-104 and Ser-106 [24]. A physiologically relevant dose of E2, 10^{-8}M was selected for the subsequent experiments. E2 (10^{-8}M), up-regulated the known E2 regulated gene, PR mRNA by 24h (Fig. 2G), as expected. Supplementing the medium containing 10% FBS with estrogen did not augment BrdU incorporation (Supplementary Fig. S3B & S3C). FBS, stripped of steroid hormones by charcoal (CSFBS), was used in subsequent experiments in order to elicit an estrogen-dependent proliferative response. Cells were maintained in CSFBS for at least 48 hours prior to challenging with estrogen in CSFBS. Of the fetal bovine concentrations tested only 2% CSFBS with estrogen had a biologically significant dose dependent effect on proliferation as assessed by MTT assay (Fig. 2E & Supplementary Fig. S3D). This was preferable to

using the serum free medium which changed ISK morphology, detaching from the monolayer and significantly compromised the cell survival. Unless for a very short-duration experiments (<12h) we did not find serum-free conditions to be suitable.

Chemosensitivity

Doxorubicin is a chemotherapy drug used to treat many types of cancer. The cytotoxic effect of doxorubicin on ISK and RL95-2 cells was examined to ascertain, the most suitable concentration to use in the CAM model. The IC50 for doxorubicin on ISK cells after 72h treatment as assessed by MTT assay was approximately 0.2µM (Fig. 3A). Spheroids of ISK in 3D cultures disintegrated with 0.1µM doxorubicin treatment for seven days (Supplementary Fig. S4) and this was associated with an increase in active caspase 3 levels (Fig. 3A). The IC50 for RL95-2 after 96 hours was approximately 0.03µM. (Fig. 3B). After 72h treatment, no IC50 was reached for RL95-2 as assessed by MTT assay. With 1µM Doxorubicin treatment the MTT analysis showed significant differences between time points, ($p<0.0001$), FACS analysis also demonstrated a 60% reduction of the live cell population after 72h treatment with 10µM of doxorubicin as a secondary method to assess the cytotoxic effect of doxorubicin on RL95-2. Both assays showed similar effects on viability (Fig. 3B & 3C).

Assessing changes functional characteristics

Cell proliferation, migration and invasion are commonly evaluated functional attributes in cancer research. However, the exact experimental method suitable for each cell line may vary, therefore further optimisation is required.

Assessing changes in cell migration and invasion *in vitro* and *in vivo*

Cell proliferation can be assessed *in vitro* using experimental methods such as BrdU incorporation (Supplementary Fig. S3), MTT assay, FACS analysis (Fig. 3B & 3C) as we have already demonstrated in sections above.

Migratory ability

Migration of cancer cells to seed extra-uterine sites, giving rise to metastatic lesions, is an important feature and can be assessed *in vitro* with the 'scratch' or 'transwell' assays [25]. Our optimisation of the scratch assay demonstrated the importance of prior knowledge of the 2D growth of cells. Scratch assay depended on the growth/migration of the cells in a horizontal plane, thus was only suitable for ISK and HEC1A cells. RL95-2 and MFE280 were unable to close the scratch even after 72h. Instead, they displayed a multi-layered growth of cells growing on top of the adherent monolayer (Fig. 4D). Therefore, to illustrate the utility of this method to assess migration, we compared ISK with HEC1A cells and developed an ImageJ-based analysis of the percentage reduction of the scratched area. In HEC1A the scratched area was recovered within 48h, whereas the ISK cells still had 40% of the scratch area exposed after 48h (Fig. 4A+4B).

We then illustrated migration using transwells, an alternative technique, and compared HEC1A cells with RL95-2 cells. HEC1A cells readily migrated through the transwell membrane without preconditioning, whereas preconditioning was required for RL95-2 migration. Under similar conditions, the HEC1A cells migrated at an increased rate compared with RL95-2 cells (Fig. 4E+4F).

Invasion

In vivo assessment of invasion

The invasion and metastatic ability of cancer cells can be ideally assessed in a multicellular in vivo system, and here we demonstrate the use of a relatively inexpensive, reproducible and rapid animal model system to examine EC cell invasion. Similar to the above *in vitro* migratory studies, cells of different invasive potential, ISK, HEC1A and RL95-2 were tested *in vivo* by growth in the CAM model for seven days, demonstrating a clear difference between the generated-tumours (Fig. 5B). ISK cells grew as sheets on CAM with no invasion or induction of neovascularisation determined by visualising chick vasculature within tumours. In contrast, large, macroscopic tumours were produced by both HEC1A and RL95-2 cells inducing a visible complex of tumour-associated blood vessels (Fig. 5B). We also demonstrate that the chemosensitivity of the tumours generated can be tested in this model where 72h treatment from day 11-14 with doxorubicin on the visible RL95-2-generated tumours affected viability, decreased proliferation (Ki67), and increased apoptosis (Bcl-2, Bax) as observed on tissue sections of paraffin-embedded samples (Fig. 5D).

Discussion

We demonstrate that EC cell lines could be utilised to model the important *in vivo* features of EC cells, a proliferative response to E2 *in vitro*. In doing so, we also developed a practically useful methodological flow chart (Fig. 6), to identify the best EC cell line to answer different research questions. We believe that initial use of this flow chart; will ensure selection of the best cell line, and most appropriate methodology to produce robust data, while saving time and resources. Our work underscored three important areas that are vital steps *in vitro* studies using EC cell lines; (1) authentication of the cell line, (2) prior establishment of pre-conditioning requirements

to elicit a response in an individual cell line, and (3) establishing baseline characteristics and growth pattern.

In vitro culture of primary human endometrial epithelial cells from normal and carcinomatous tissue is a challenging process; consequently, the number of established EC cell lines that are currently commercially available to all researchers worldwide is also limited, and there is a complete absence of model normal/benign human endometrial epithelial cell line(s).

Authentication of the cell lines, using STR profiling ensures that the features of the cell line have not changed, particularly when they are obtained from other sources than the reputable, authenticity guaranteed suppliers. We highlight the importance of initial genomic characterisation by demonstrating a significant genetic drift in the old HEC1A. This could be due to the cross-contamination with other cell lines, which has become a prolific problem throughout the world, with an alarming estimation that 15% of cell lines utilised in the USA to be either misreported or contaminated with other cell lines [26]. Although the scientific community is slowly combating the problem, caution needs to be taken when interpreting results from publications that have not authenticated the cell lines used. In the context of EC research, over the last decade, at least six publications used the so-called hormone responsive hTERT-EEC cell line, which was the misidentified breast cancer cell line MCF-7 [27-30]. Although the classical proliferative response to estrogen is seen in breast and endometrial tissue, the two tissues have very different responses to some other hormonal agents such as progestogens and tamoxifen [31, 32]. Therefore, the presumed clinical relevance of *in vitro* studies using hTERT-EEC may not have been completely relevant to the endometrium. This example highlights the importance of fully understanding the model being used. Obtaining cell lines from authorised cell banks, which authenticate cell

lines prior to purchase, will alleviate concerns of receiving misidentified cells whilst confirming the profile remains static throughout the study and at the end of the experimental period is also important.

The published literature is sparse in describing the indispensable steps in assessing proliferative response to hormones (E2) of EC cell lines *in vitro*, such as preconditioning of the cells [33]. ERα was still phosphorylated in the presence of 10% FBS with E2, but the proliferative response was only observed at lower CFBS concentrations. Our results are in keeping with the findings of Holinka et al. [33]. The baseline levels of ERα phosphorylation were different with CSFBS and FBS that demonstrates the importance of establishing baseline conditions with which to compare the treatment response, without masking them by endogenous agents within the culture model. Under normal culture conditions, EC cell proliferation depends on FBS to provide the essential growth factors, amino acids, etc., therefore, unless the cells are established in a baseline, non-enriched conditions prior to the experimental process, they are unable to demonstrate the total response to the growth stimuli such as E2. Cells have to be initially maintained under optimal growing conditions and then primed (CSFBS) prior to challenge (E2). Furthermore, FBS is an unknown entity with dramatic differences between batches with unquantified concentrations of the components. Therefore, it is important to conduct all experiments using the same batches of FBS to reduce variability across experimental replicates. Using CSFBS to maintain cell viability during the experimental process will reproducibly prime the cells to respond to the hormonal treatment, however, sub-optimal conditions might select for cells with reduced nutrient requirements. Thus, our work highlights the importance of the optimal preconditioning for the cell line of choice in endometrial research, for translational research in hormone modulation studies *in vitro*.

Examining cell lines for the essential apparatus to respond to a signal of interest is pivotal and our work demonstrated the importance of characterising the cell lines for the expression of ERα before further functional studies. Since even the high grade advanced ECs retain some hormone responsiveness [16], *in vitro* or *in vivo* models that mimic them are important in EC research.

MTT assays work under the assumption that only living cells will be able to metabolise the MTT into formazan, however several other parameters such as the drugs used, the components of the culture medium can alter the metabolism as well as viability in these assays [34]. However, they do offer a quick, low-cost indirect method to assess cell death or cell proliferation to prompt further investigation. However, we recommend confirmation using another method that is not reliant on cell metabolism. FACS offers a multifaceted tool in which multiple parameters can be assessed depending on the dyes used.

In our migration experiments, we demonstrated that the initial identification of the growth pattern of a suitable cell line dictates the potential subsequent experimental plan. We assessed cellular functionality using several methods considering the cell line specific differential growth pattern, for example, the scratch assay was only suitable for cells that grow in a horizontal 2D plane. The migratory ability of cells is an important factor when studying the metastatic spread, facilitating discovery of novel therapeutics. Transwell assay was suitable for the RL95-2 cells which displayed multi-layered 2D growth but required strict adherence to protocol to reduce variance. Therefore, initial assessment of the exact growth conditions and patterns for each of the cell lines being studied is vital to obtain reproducible data.

Many animal models have been employed to assess cancer-associated angiogenesis and metastasis *in vivo*. Of these, the CAM model has the advantage of being a relatively cheap, widely available, rapid and high throughput model and although it has been previously used to assess metastasis and invasion in ovarian cancer cell lines [35] and benign endometrial tissue [36], we have used this model in the context of EC cell lines for the first time. Upon inoculating onto the CAM, the EC cell lines would be severely depleted of growth factors and would not survive long unless the cells invade into the CAM and establish a vascular supply using the host (chick) vasculature, therefore the assessment of the cell viability and apoptosis within these newly formed tumours will reflect the potential aggressiveness of the EC cells. Harvested tumour established on the CAM can be analysed for markers of proliferation or apoptosis with IHC or qPCR. We have illustrated that in this model, the well-differentiated ISK cell line which showed lower invasive features *in vitro*, was unable to produce significant tumours whereas the less differentiated HEC1A and RL95-2 cells with more invasive *in vitro* behaviour produced large tumours with an impressive neo-vascularisation.

Conclusions

Cell lines offer a unique platform to gain insight into the molecular processes occurring *in vivo*; however careful selection of cell lines is important not only to be able to extrapolate the data into the clinical context but to compare with the previous and future studies. From the perspective of EC research, due to the limited *in vitro* capacity of primary cells, they can be used initially for rapid, reproducible discovery projects without relying on precious yet typically heterogeneous patient tissue. We have shown that essential feature of EC, estrogen induced proliferation can be demonstrated with necessary precondition steps in ISK cell line and we propose a pragmatic

methodological guide that will facilitate robust data generation in projects using cell lines, which saves time and resources.

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Conflict of Interest

No conflict of interests to declare.

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Figure Legends

Figure 1. A) STR profiles for gifted HEC1A and ATCC sources HEC1A cells with electropherograms for both analyses, illustrating an example of stutters in brackets,

omitted peaks in italics and additional peaks underlined compared with the published profile. B) Micrographs of Ishikawa (ISK), HEC1A, RL95-2 and MFE 280 cells in culture. C i) ISK grown in Matrigel™ ii) ISK grown in Matrigel™ stained with DAPI iii) Cross-section images of ISK spheroid grown in Matrigel™ stained for pan cytokeratin (PanCK) and iv) DAPI showing the filled spheroid structure.

Figure 2. *Hormone receptor expression*. A) Estrogen receptor α (ERα), Estrogen receptor β (ERβ), Progesterone receptor (PR) and Androgen receptor (AR) mRNA expression by qRT-PCR in the four endometrial cancer cell lines; Ishikawa (ISK), HEC1A, RL95-2 and MFE280 B) ERα and ERβ expression using antibody staining by IHC and IF in the ISK cell line. C) The expression of ERα protein using western blot for the cell lines ISK, HEC1A, RL95-2 and MFE280. D) PR and AR expression in 4 cell lines determined by IHC, positive staining shown by brown colour counterstained with haematoxylin (blue). Nuclear detection of the receptors was the criterion for being called positive or negative expressing. E) MTT assay: ISK cell proliferation was induced by 10^{-8}M E2 or EGF treatment for 72h; E2 induced a marginal increase in proliferation with 5% CSFBS whilsta substantial induction of proliferation was observed when media containing 2% CSFBS was used, However, no response was observed with E2 treatment in the presence of 10% FBS. Statistical analysis using an independent sample t-test ****= $p<0.0001$, *= $p<0.05$. EGF treatment was used as a positive control. F) ERα total and phosphorylated protein increased in ISK cell line with treatment of 10^{-8}M 17β-estradiol (E2) for 72h. G) mRNA level of PR after treatment with 10^{-8}M E2 compared with vehicle t-test ***= $p<0.005$. Similar to the benign normal endometrial epithelial cells [37] and the low grade EC [16] the well differentiated ISK cell line expressed mRNA and protein for all 4 steroid receptors (ERα, ERβ, AR and PR) studied. The less well differentiated RL95-2 and MFE280 cell lines exhibited

loss/heterogeneous pattern of nuclear AR and PR, whilst more invasive, moderately differentiated HEC1A cell line also showed loss of AR and PR. These data illustrates the differences between each of the cell lines that potentially provide appropriate models to simulate various differentiation stages of EC in the laboratory.

Figure 3. A) Percentage survival of ISK cells treated with Doxorubicin (DOXO) (0.01,0.1 and 1µM) for 72h in 2D growth, and 3D culture in Matrigel™ treated with 0.1µM DOXO and Vehicle (V) for 72h. IF stained spheroid treated with doxorubicin shows increased activated caspase 3 (red color counterstained with DAPI). Western blot showing increased activated caspase 3 in doxorubicin treated cells. . B) Dose response of RL95-2 cell line to DOXO treatment from 24-96h of treatment with a range of doses 0.0001-10µM compared to control cells treated with V (DMSO) each time point using MTT assay. With DOXO treatment at 1µM showed significant differences between 24h and 96h. C) FACS analysis of 7-AAD and Hoechst 33342 dual stained RL95-2 cells treated with 1µMDOXO or V (DMSO), showed significant changes in the percentage of live, necrotic and dead cell populations. Data shown as percentage of cells detected per quadrant compared to single stained controls. Error bars represent SEM. Significance was determined using t-test. ****= $p<0.0001$, **= $p<0.005$ and *= $p<0.05$.

Figure 4. A) Scratch area measured at set time points after scratch in ISK and HEC1A cell lines. 6 replicates per time point and error bars indicate SEM. B) Representative images of scratch closure images from 0-48h. The HEC1A cell line completely recapitulated the scratch area between 24-48h whereas ISK had not C) Example segmentation image of the MitoBio ImageJ plug in analysis to measure scratch area on ISK. D) MFE280 cell line grew upward around scratch edges preferring to grow on top of one another making measurement along the horizontal plane unrepresentative

of the cell lines migratory capacity. RL95-2 cells took much longer to form confluent monolayers (requiring larger number of cells to be plated) and the edges of the scratch would lift away from the plate surface. Tranwell migration was used to assess RL95-2 migration in comparison with HEC1A. E) Number of HEC1A and RL95-2 cells that migrated through a transwell insert after 24h incubation after 16h preconditioning in serum free media toward a 10% FBS chemoattractant in cell line media (t-test **p=0.0024). F) Image of inverted transwell insert after cells have migrated through the membrane.

Figure 5. 3D culture models. A) Timeline for Chorioallantoic Membrane Model (CAM) assay. E0 indicates day eggs were incubated in the hatchery. At E3 excess albumen removed using an egg punch and a sterile syringe allowing windows to be cut in the uppermost side of the egg shell. At E7 CAMs were inoculated with cell lines and incubated for a further 7 days. For drug or vehicle control treatments Eggs were injected into the chorioallantoic sac at E11 and E13 with either DMSO (4µL/egg) or Doxorubicin (*3*µM) diluted in PBS. At E14 tumours were imaged and excised, fixed in 10% NBF and paraffin wax embedded. B) HEC1A, ISK and RL95-2 grown on CAM. C) Cell lines grown in Matrigel™ D) IHC of excised tumours of RL95-2 tumours grown in CAM model sectioned and stained for Ki67, Bax and Bcl-2 after treatment for 72h with Doxorubicin (DOXO) or DMSO (V) as vehicle control.

Figure 6. Example workflow for endometrial cancer cell line projects. Tumour weight refers to mice studies rather than CAM.

Supplementary Figure Legends

Supplementary Table S1. List of commercially available cell lines of endometrioid origin, cell lines of unverifiable origin were excluded and additional information was added from source databases. Adapted from Barretina et al. 2012 [17] and populated with additional information from the internet search of commercial suppliers of cell lines. Source; American Type Culture Collection (ATCC), German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) (DSMZ), Japanese Collection of Research Bioresources Cell Bank (JCRB), Bioresources of RIKEN (RIKEN) and The European Collection of Cell Cultures (ECACC). Cell lines of unknown tissue origin were excluded.

Supplementary Table S2. STR profiles for the endometrial cancer cell lines; HEC1A-COL and HEC1A-ATCC, Ishikawa (ISK), RL95-2, MFE280 cell lines after completion of experiments to confirm the cell line identities. Peaks which are additional to the published profile are in green and omitted peaks are in red. Profile matches from 3 STR profile databases (DSMZ, ATCC and the location the cells were sourced from) are shown for each cell line.

Supplementary Figure S3. 17β-Estradiol (E2) effect on ERα and phospho-ERα. A) Western blot of phosphor-ERα (Ser118) after 72h E2 treatment at a range of concentrations 10^{-6}-10^{-9}M with a GAPDH control. B) BrdU incorporation with 4h treatment of 10^{-8}M E2 with 5% charcoal stripped FBS (CSFBS). C) Example images of BrdU incorporation in response to E2 with DAPI counterstaining. ISK cells grown in DMEM:F12, phenol-red free media with 5% CSFBS $5x10^3$ cells seeded per well in 8 well slide chamber with vehicle or E2 10^{-8}M (Sigma) for 2 h. BrdU (5'bromo-2'deoxyuridine, #B5002, Sigma) added to make 10μM concentration. Incubated at

37ºC for 4 h. Cells washed with PBS and fixed with 10% NBF. Cells denatured with 2M HCl for 1 h, washed in borate buffer and blocked with 2.5% normal horse serum (Vector labs) for 30 min at room temperature. BrdU incorporation detected using Mouse monoclonal anti-BrdU antibody 1:200 incubated overnight and followed by Alexa Fluor 488 secondary antibody incubation. Cells were then washed and mounted using Vectashield with DAPI (Vector labs). D) Ishikawa cell treated with 10^{-8}M E2 in media supplemented with 5% Charcoal Stripped FBS assessed for proliferative response by the MTT assay. Showing no proliferative response to E2 compared with vehicle (V), EGF used as a positive control. The 5% CSFBS media was not able to prime the cells to respond to E2 detected by BrdU incorporation or MTT assay.

Supplementary Figure S4. Ishikawa cells grown in Matrigel™ treated with either vehicle, or with increasing doses of Doxorubicin (0.01µM, 0.1µM, 1µM) for 3 days and stained with DAPI. ISK tumour spheres were viably unaffected with 0.01µM Doxorubicin treatment, but some disaggregation of the tumour spheres started to occur at 0.1µM dose and the spheres completely disintegrated with 1µM Doxorubicin treatment..

Supplementary Table S5. PCR Primer Table. Including primer sequences as provided by the literature cited, amplicon size in base pairs, primer efficiency and a publication or source reference.

Supplementary Table S6. Antibodies Table. *Heat induced antigen retrieval by pressure cooking in citrate buffer pH 6. [1] Ely, Cambridgeshire, UK; [2] Cambridge, UK ; [3] Oxford, UK ; [4] Newcastle upon Tyne, UK [5] Dorset, UK ; [6]Hitchin, Hertfordshire, UK. O/N = Overnight, ICC= Immunocytochemistry, WB= Western Blot.

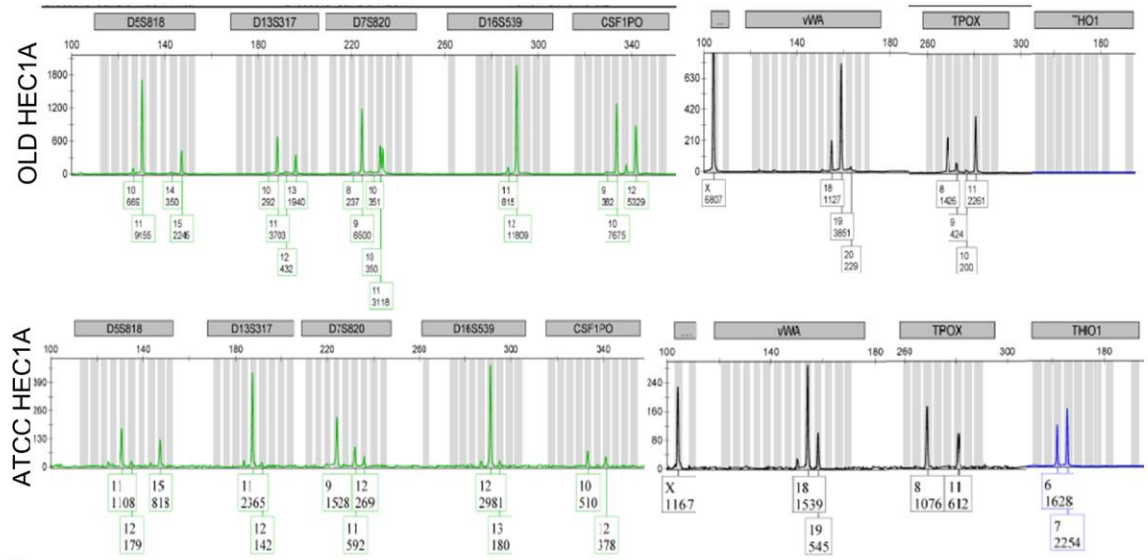
Supplementary Table S7. Table of Cell Line Characteristics for Ishikawa, HEC1-A, RL95-2 and MFE280. Hormone Receptor Status determined by immunohistochemical staining (Figure 2.). Doubling time determined by Goto et al 2008 [38], Kuramoto and Nishida 2012 [39], Way et al. 1983 [11], and DSMZ MFE280 cell line datasheet [40] .

682 Figure 1

A

Cel \ Loci	D5S818	D13S317	D7S820	D16S539	CSF1PO	A	vWA	TPOX	TH01
HEC1A – COL	(10) 11,*14*,15	11,*12*, 13	(8)9, (10),11	11,12,*13*	(9),10,12	X	18,19, 20	8,*9*,*10*,11	6,7
HEC1A-ATCC	11,12,*14*, 15	11,12	9,11,12	12,13	10,12	X	18,19	8,11	6,7

omitted peaks (italics) /additional peaks (underlined)/ brackets indicate stutter



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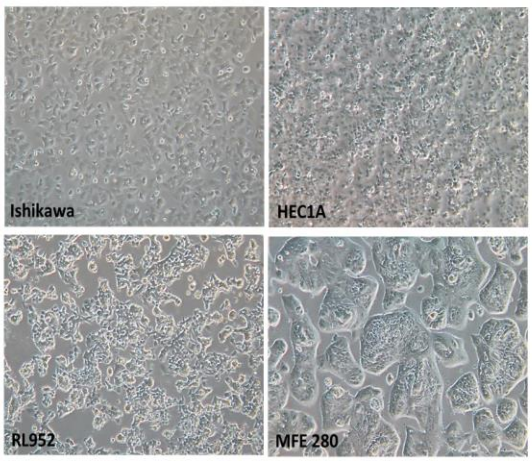
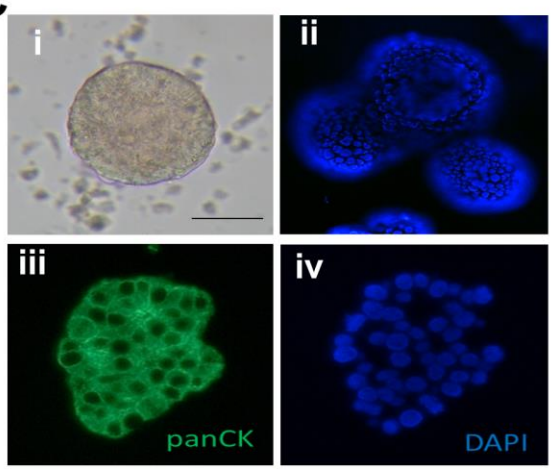


C



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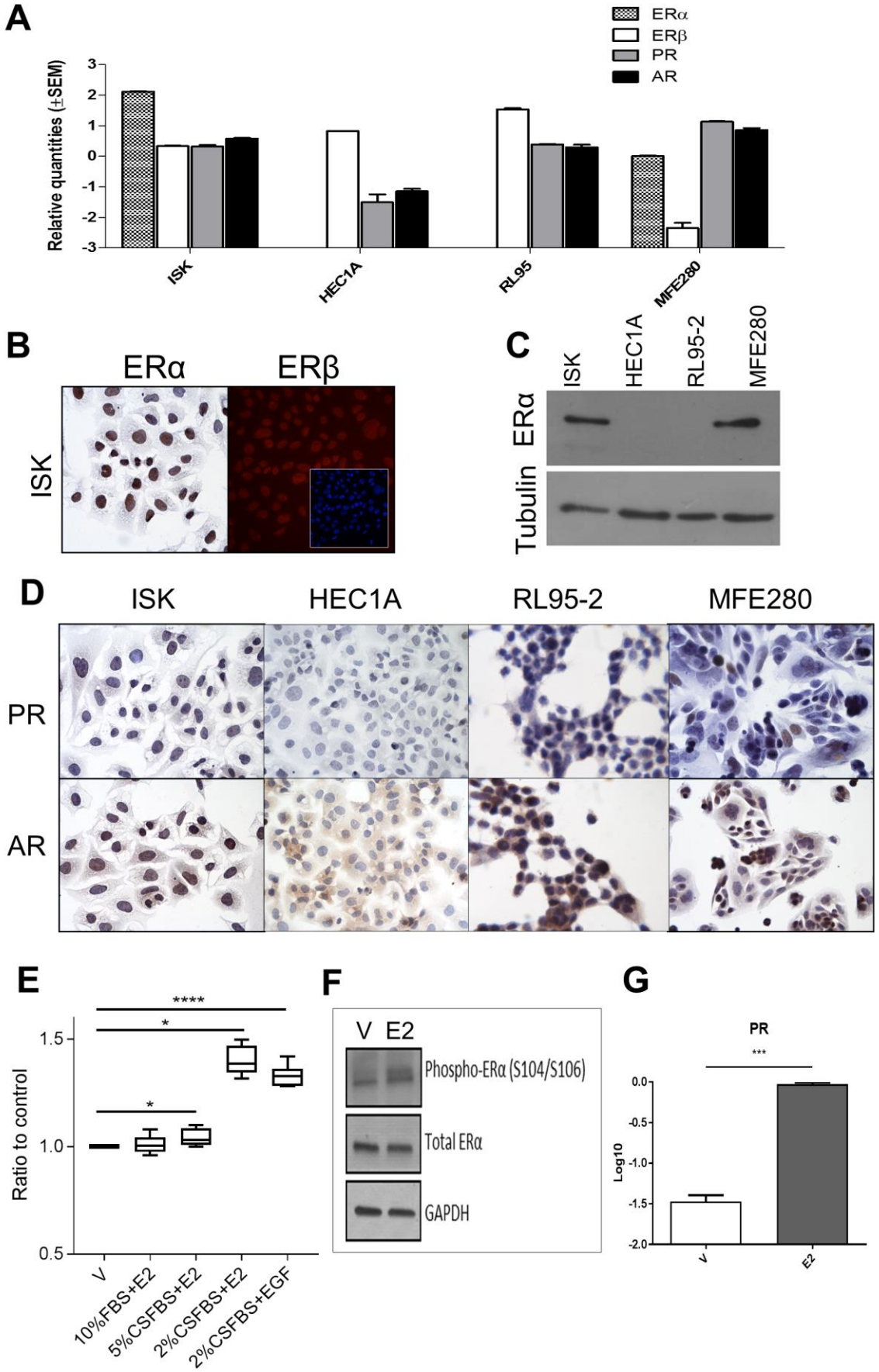
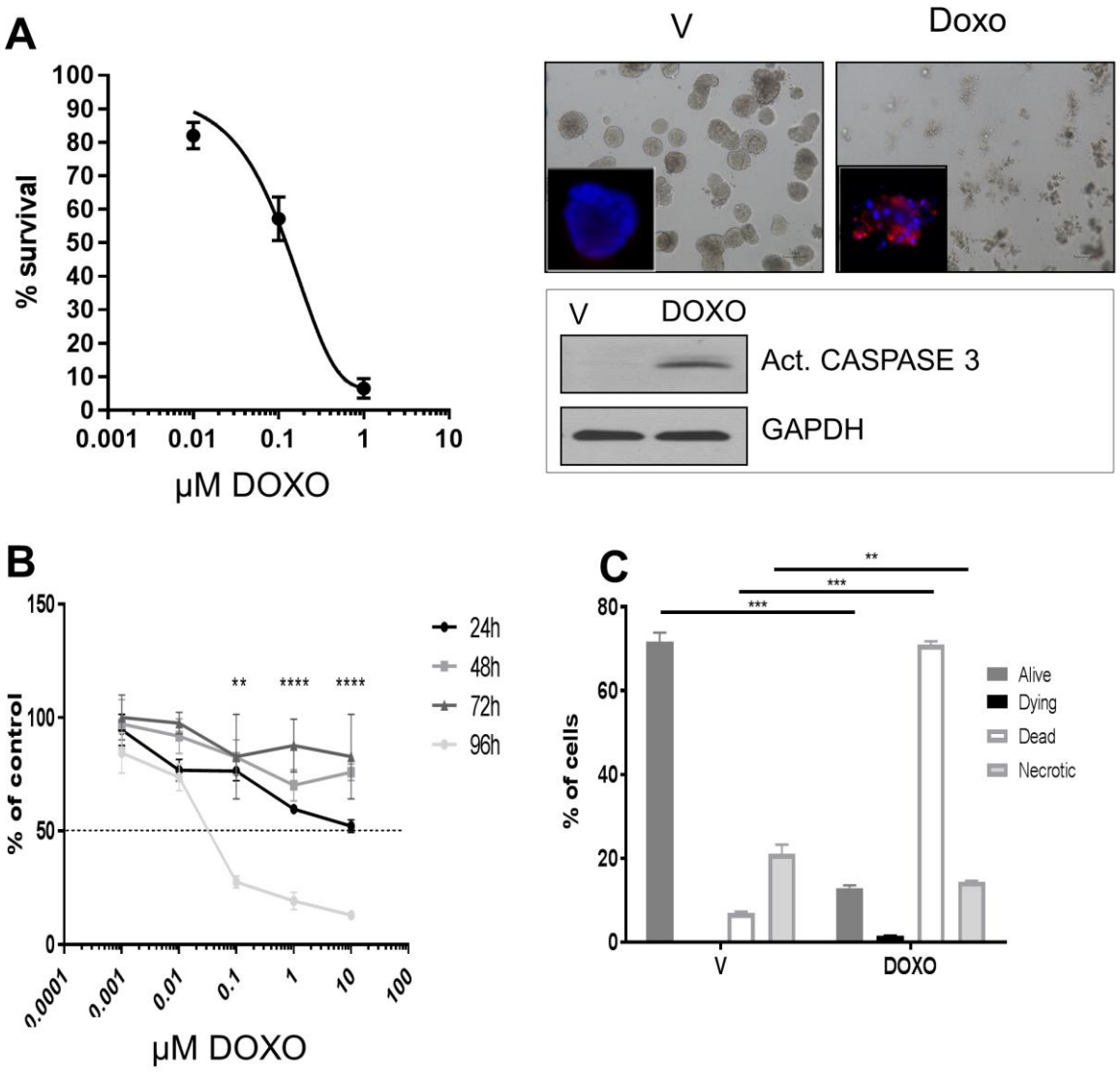
686 Figure 2

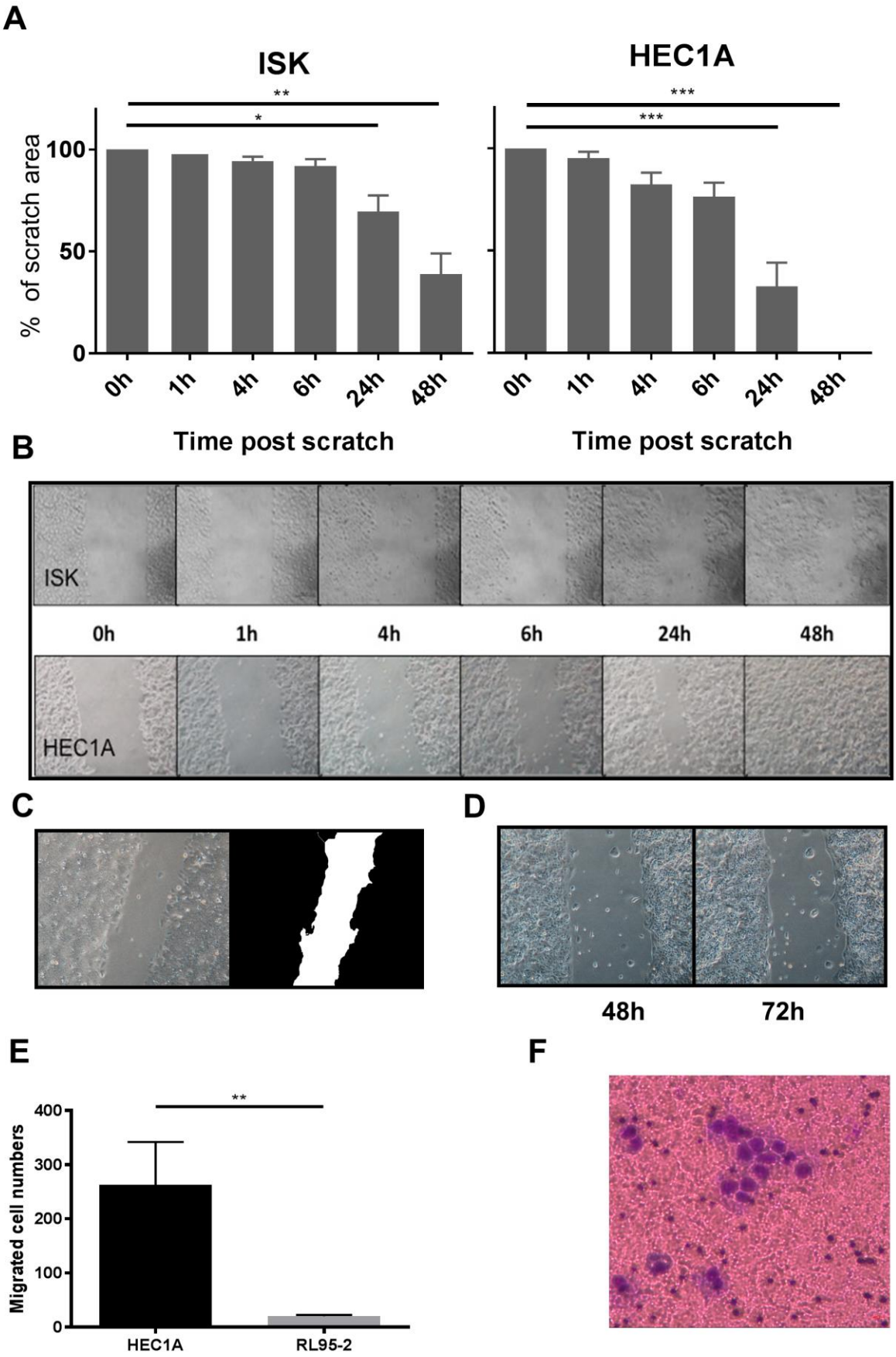

A
ERα
ERβ
PR
AR
Relative quantities (±SEM)
3
2
1
0
-1
-2
-3
ISK
HEC1A
RL95
MFE280
B
ERα
ERβ
ISK
C
ISK
HEC1A
RL95-2
MFE280
ERα
Tubulin
D
ISK
HEC1A
RL95-2
MFE280
PR
AR
E
****
*
*
Ratio to control
1.5
1.0
0.5
V
10%FBS+E2
5%CSFBS+E2
2%CSFBS+E2
2%CSFBS+EGF
F
V E2
Phospho-ERα (S104/S106)
Total ERα
GAPDH
G
PR
***
Log10
0.0
-0.5
-1.0
-1.5
-2.0
V
E2

687

Figure 3


A
% survival
μM DOXO
V
Doxo
V
DOXO
Act. CASPASE 3
GAPDH
B
% of control
μM DOXO
24h
48h
72h
96h
C
% of cells
Alive
Dying
Dead
Necrotic
V
DOXO

695 Figure 4


A
ISK
HEC1A
**
*
***
***
% of scratch area
100
50
0
0h
1h
4h
6h
24h
48h
Time post scratch
Time post scratch
B
ISK
0h
1h
4h
6h
24h
48h
HEC1A
C
D
48h
72h
E
**
Migrated cell numbers
400
300
200
100
0
HEC1A
RL95-2
F

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697 Figure 5

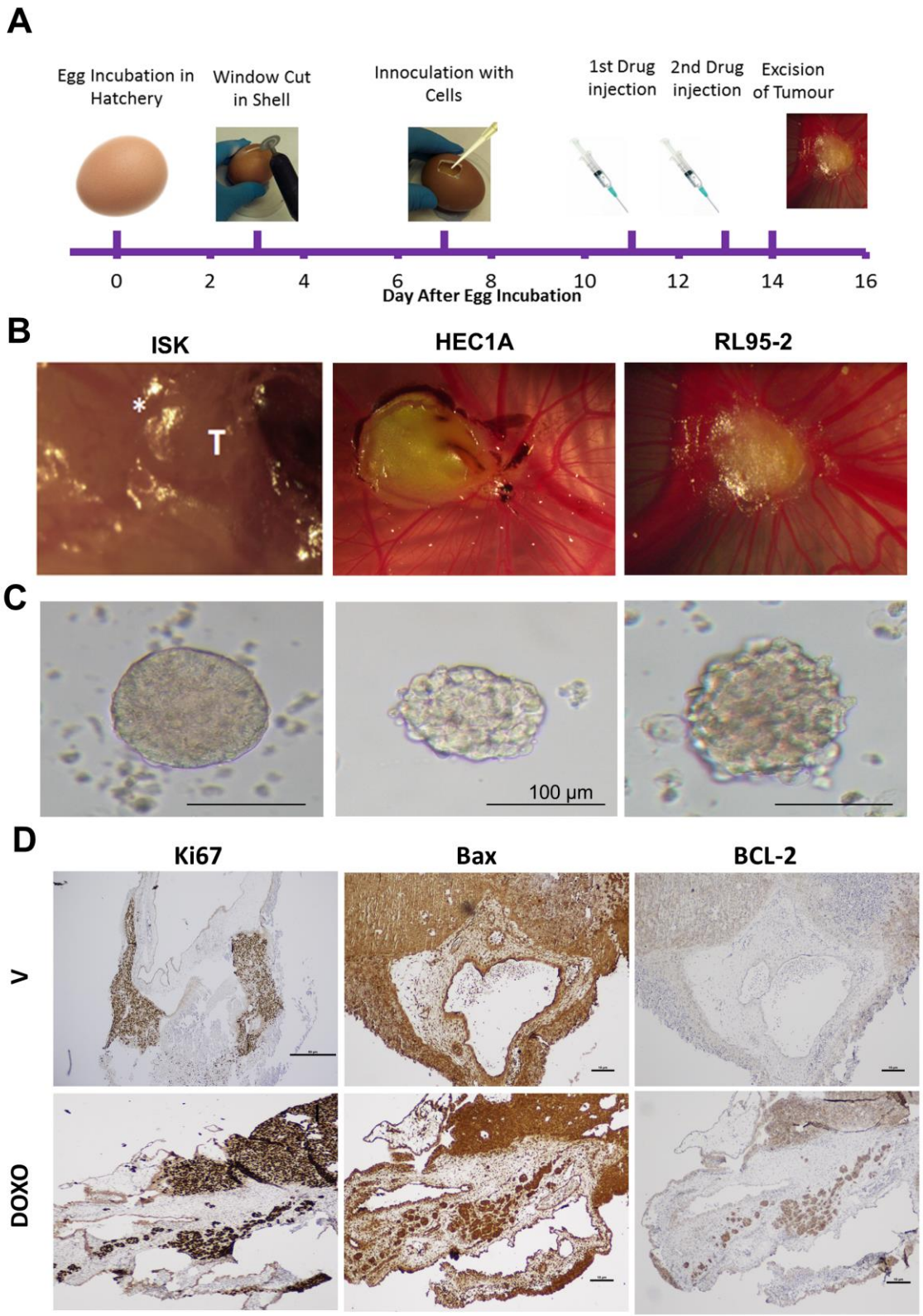

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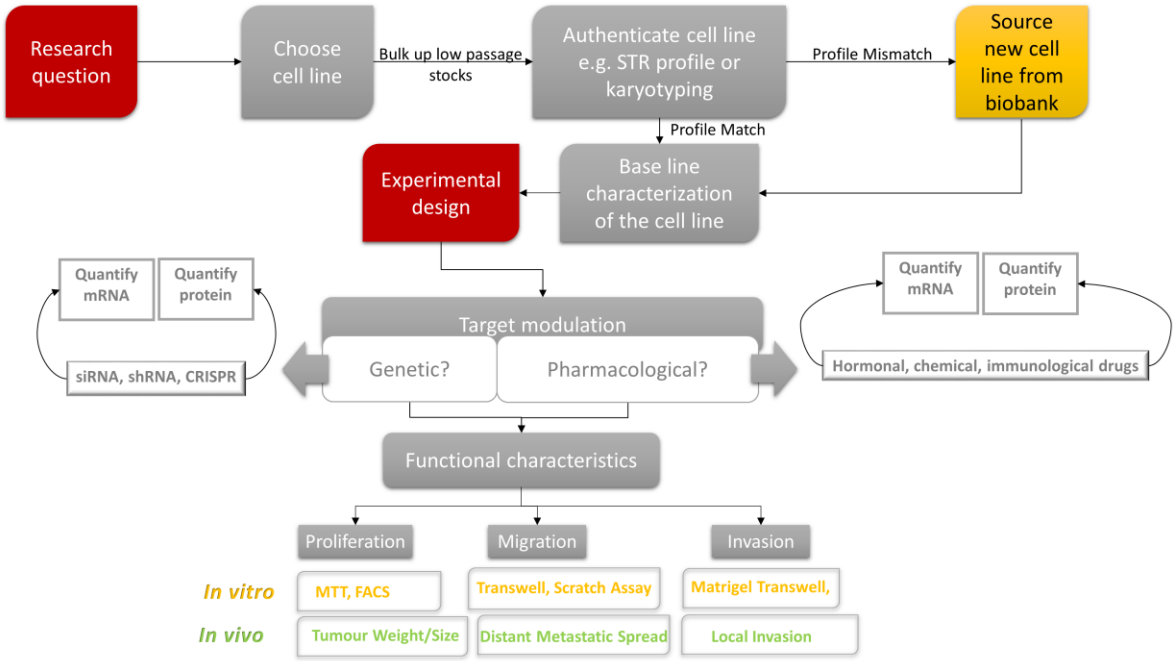
Figure 6


Research question
Choose cell line
Bulk up low passage stocks
Authenticate cell line e.g. STR profile or karyotyping
Profile Mismatch
Source new cell line from biobank
Profile Match
Experimental design
Base line characterization of the cell line
Quantify mRNA
Quantify protein
siRNA, shRNA, CRISPR
Target modulation
Genetic?
Pharmacological?
Quantify mRNA
Quantify protein
Hormonal, chemical, immunological drugs
Functional characteristics
Proliferation
Migration
Invasion
In vitro
MTT, FACS
Transwell, Scratch Assay
Matrigel Transwell,
In vivo
Tumour Weight/Size
Distant Metastatic Spread
Local Invasion

712 Supplementary Figures

Supplementary Table S1.

Cell Line	Tissue Diagnosis/	Ethnicity	Age (y)	SOURCE #	SOURCE	Synonyms	NOTES
AN3 CA	Endometrioid	Caucasian	55	HTB-111	ATCC	--	Derived from a metastatic lesion in the lymph node of patient with endometrial carcinoma alerted to the condition by onset of the malignant disorder acanthosis nigricans.
EFE-184	Carcinoma	Caucasian	69	ACC 230	DSMZ	--	Established from the ascitic fluid of a patient with endometrial carcinoma relapse in 1985. 92h doubling time, epitheliod cells growing in
HEC-108	Endometrioid	Japanese	Unknown	JCRB1123	JCRB	--	Established from an endometrial adenocarcinoma. Deposited in 2005, cell line with epithelial-like morphology. Nude mouse-transplanted endometerial
HEC-116	Endometrioid	Japanese	Unknown	JCRB1124	JCRB	--	--
HEC-151	Endometrioid Grade 2	Japanese	Unknown	JCRB1122	JCRB	--	Human endometrioid adenocarcinoma deposited in
HEC-155	Endometrioid	Unknown	Unknown	JCRB1127	JCRB	HEC-155, HEC-180	No longer available http://www.ncbi.nlm.nih.gov/
HEC-1-A	Endometrioid	Unknown	71	HTB-112	ATCC	HEC-1, HEC-1-A, HEC-1-B, NCI-H1573	HEC-1-A and HEC-1B are subclones of HEC-1 and share a high SNP identity. Cells established in 1968 from a moderately well differentiated adenocarcinoma, cells form typical papil-
HEC-1-B	Endometrioid	Unknown	71	HTB-113	ATCC	HEC-1, HEC-1-A, HEC-1-B, NCI-H1573	
HEC-251	Endometrioid	Japanese	Unknown	JCRB1141	JCRB	--	Epithelial like morphology established in 2005
HEC-265	Endometrioid Grade 1	Japanese	Unknown	JCRB1142	JCRB	--	Epithelial like morphology from uterus corpus deposit-
HEC-50B	Endometrioid Grade 3	Japanese	Unknown	JCRB1145	JCRB	--	Human endometriod adenocarcinoma patient deposited in 2005 with epithelial-like
HEC-59	Endometrioid Grade 2	Japanese	Unknown	JCRB1120	JCRB	--	Human tumour cell line from endometrioid adenocarcinoma deposited in 2005 with epithelial-like morphology

713

Cell Line Name	Tissue Diagnosis/ Grade	Ethnicity	Age (y)	SOURCE #	SOURCE	Synonyms	NOTES
HEC-6	Endometrioid	Japanese	Unknown	JCRB1118	JCRB	--	Human endometrial adenocarcinoma cell line with epithelial like morphology deposited in 2005.
HEC-88nu	Endometrioid	Japanese	Unknown	JCRB1121	JCRB	--	Epithelial like morphology
HOUA-I	Endometrioid	Unknown	55	RCB0659	RIKEN	--	Poorly differentiated adenocarcinoma. Cell growth is slow. Epithelial-like morphology
Ishikawa	Endometrioid	Unknown	39	99040201	ECACC	Ishikawa, ECC-1	http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=99040201&collection=ecacc_gc
Ishikawa (Heraklio) 02 ER-	Endometrioid	Unknown	Unknown	98032302	ECACC	--	--
JHUAS-1	Adenosquamous Carcinoma	Japanese	56	RCB1544	RIKEN	--	Epithelial like morphology
JHUAS-2-L	Adenosquamous Carcinoma	Japanese	Unknown	RCB1545	RIKEN	--	B lymphocyte isolated from a japanese adenosquamous carcinoma
JHUCS-1	Carcinoma	Japanese	57	RCB1547	RIKEN	--	Established from a mixed mesodermal tumour
JHUEM-1	Endometrioid Grade 2	Japanese	56	RCB1548	RIKEN	--	Epithelial like morphology
JHUEM-14	Endometrioid	Japanese	44	RCB2225	RIKEN	--	Epithelial like morphology
JHUEM-2	Endometrioid	Japanese	62	RCB1551	RIKEN	--	Small angular cellular morphology
JHUEM-3	Endometrioid	Japanese	60	RCB1552	RIKEN	--	Epithelial-like morphology
JHUEM-7	Endometrioid	Japanese	58	RCB1677	RIKEN	--	Epithelial like morphology
KLE	Endometrioid	Caucasian	64	CRL-1622	ATCC	--	Epithelial-like morphology
MFE-280	Endometrioid Grade 3	Unknown	77	ACC 410	ECACC	--	Established from a recurrence of endometrial carcinoma (adenomatous, grade 3) in 1990; described as forming heterotransplantable tumours in nude mice and as carrying progesterone receptors. Epitheliod cells growing adherently in monolayers. 60-90h doubling time.

Cell Line Name	Tissue Diagnosis/ Grade	Ethnicity	Age (y)	SOURCE #	SOURCE	Synonyms	NOTES
MFE-296	Endometrioid Grade 2	Caucasian	68	ACC 419	DSMZ	--	Established from moderately differentiated endometrial adenocarcinoma in 1991; cells were described to express androgen receptors; cells were described to be tumorigenic nude mice. Epithelial-like polymorphic cells growing adherently in monolayers. Doubling time 50-60h.
MFE-319	Endometrioid Grade 1/2	Unknown	81	ACC 423	DSMZ	--	Established from the primary adenosquamous endometrium carcinoma in 1992; described to form poorly differentiated tumours in nude mice. epithelial-like adherent cells growing as monolayer. Doubling time 100h.
OMC-2	Endometrioid	Japanese	59	RCB2830	RIKEN	--	Cell growth is slow.
RL95-2	Adenosquamous Carcinoma	Caucasian	65	CRL-1671	ATCC	--	Grade 2 endometrial adenocarcinoma
SNG-II	Endometrioid	Unknown	43	IFO50312	JCRB	RMUG-L, RTSG, SNG-II	SNG-II parental, RMUG-L and RTSG contaminanted/ no longer available.
SNG-M	Endometrioid	Japanese	52	IFO50313	JCRB	--	--
TEN	Clear cell carcinoma	Japanese	74	RCB1433	RIKEN	--	Epithelial like morphology.

723 **Supplementary Table S2.**

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Lab/Published STR Profile					
Loci	OLD HEC1A	NEW HEC1A	ISK	RL95-2	MFE280
A	X	X	X	X	X
D5S818	11,14,15	11,12,14,15	10,11,12	10,11	11,12
D13S317	11,12,13	11,12	9,12	8,12	10,12
D7S820	9,11	9,11,12	9,10	10	9,10
D16S539	12,13	12,13	9	11,12,13,14	11,12
vWA	18,19	18,19	14,17	16,19,20	16, 17
TH01	6,7	6,7	9,10	9,9.3	7
TPOX	8,11	8,11	8	8	9,10
CSF1PO	10,12	10,12	11,12	10,11	8,9,11,12
EV DSMZ profile database	0.83	0.95	0.91	0.92	1.0
% Match ATCC STR database	78%	94%	n/a	100%	n/a
Source Website STR information	n/a	ATCC (94% Match, 2 small additional peaks and 1 ommited peak)	ECACC (1 additional small peak)	ATCC (3 small additional peaks)	Sigma (100% Match)

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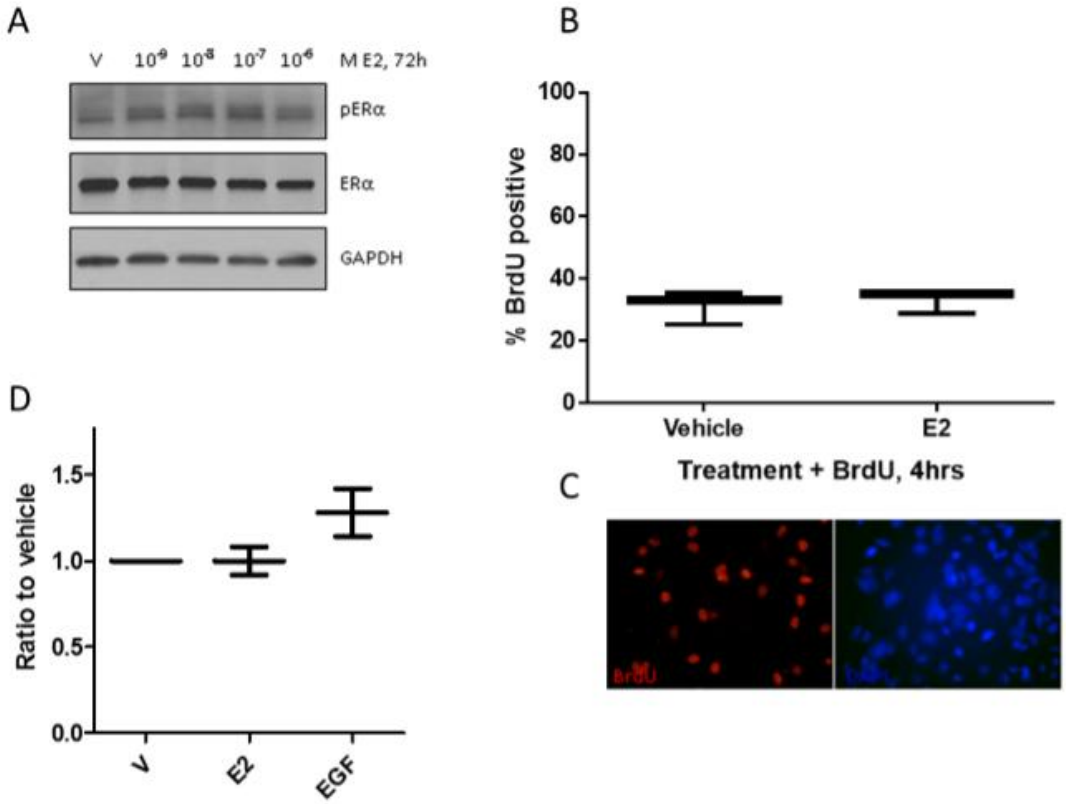
Supplementary Figure S3.



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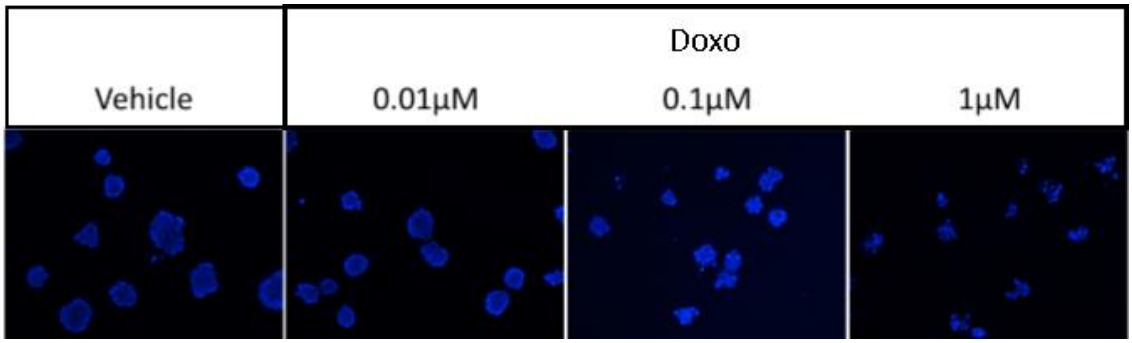
Supplementary Figure S4.



749 **Supplementary Figure S5.**

Primer	Sequence	Amplicon	Efficiency	Reference
AR	F:5'AGGATGCTCTACTTCGCCCCC3'	72	99%	Pichler et al.,2013
	R: 5'CTGGCTGTACATCCGGGAC3'			
PR	F: 5'CAGTGGGCGTTCCAAATGA3'	83	101.3%	Henderson et al., 2003
	R: 5'TGGTGGAATCAACTGTATGTCTTGA3'			
ERα	F: 5'TGATTGGTCTCGTCTGGCG3'	101	92.5%	Henderson et al., 2003
	R: 5'CATGCCCTCTACACATTTTCCC3'			
ERβ	F: 5'	87	90.3%	BioRad
	R:5'			
PPIA	5-AGACAAGGTCCCAAAGAC-3	118	96.6%	Jacob et al., 2013
	5-ACCACCCTGACACATAAA-3			
YWHAZ	F: 5'CGTTACTTGGCTGAGGTTGCC3'	69	91.1%	Marullo et al., 2010
	R:5'GTATGCTTGTTGTGACTGATCGAC3'			

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752 **Supplementary Table S6.**

Primary Antibody	Type	Assay	Clone/ Catalogue number	Supplier	HIAR* (min)	Dilution	Incubation conditions	
							Time (hour)	Temp (°C)
AR	Monoclonal	ICC	441	DAKO[1]	2	1:50	20	4
PR	Monoclonal	ICC	PgR 636	DAKO	2	1:1000	0.5	18
ERα	Polyclonal	ICC/	ab137738	Abcam[2]	2	1:50/1:1000	2	18
ERβ	Monoclonal	ICC	PPG5/10	Serotec[3]	2	1:50	20	4
Ki67	Monoclonal	ICC	MM1	Leica[4]	4	1:200	20	4
BCL-2	Monoclonal	ICC	100	Serotec[3]	2	1:100	O/N	4
BAX	Polyclonal	ICC		DAKO[1]	2	1:50	O/N	4
GAPDH	Polyclonal	WB	G9545	Sigma[5]	n/a	1:10,000	O/N	4
Phosphorylated ER (Ser 118)	Monoclonal	WB	16J4 #2511	Cell Signalling[6]	n/a	1:1000	O/N	4
Phosphorylated ER (Ser 104/106)	Polyclonal	WB	#2517	Cell Signalling[6]	n/a	1:1000	O/N	4
Anti-Pan Cy-	Monoclonal	IF	C2562	Sigma[5]	n/a	1:1000	O/N	4
Anti-Rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate)	n/a	IF	#4412	Cell Signalling[6]	n/a	1:1000	1	RT
Anti-Mouse IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 555 Conjugate)	n/a	IF	#4409	Cell Signalling[6]	n/a	1:1000	1	RT

753