

1 **Assessing estrogen-induced proliferative**  
2 **response in an endometrial cancer cell line**  
3 **using a universally applicable**  
4 **methodological guide.**

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38 Abbreviations: Endometrial Cancer (EC), Ishikawa (ISK), Estrogen Receptor (ER),  
39 Androgen Receptor (AR), Progesterone Receptor (PR), 17  $\beta$ -estradiol (E2),  
40 Polyacrylamide gel electrophoresis (PAGE), Short Tandem Repeat (STR), Charcoal  
41 stripped fetal bovine serum (CSFBS), Chorioallantoic Membrane (CAM), Neutral  
42 Buffered Formalin (NBF), Phosphate Buffered Saline (PBS), Glyceraldehyde 3-  
43 Phosphate Dehydrogenase (GAPDH), Tyrosine 3-Monooxygenase/Tryptophan 5-  
44 Monooxygenase Activation Protein, Zeta (YWHAZ), Peptidylprolyl Isomerase A (PPIA)  
45

46 **Abstract**

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

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

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

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

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

71 **Keywords** : Endometrial cancer, cell lines, estrogen

72

## 73 **Introduction**

74 Endometrial cancer (EC) is the fourth commonest malignancy in women in Europe  
75 (6% of all female cancers) [1, 2]. The increasing rates of obesity are projected to  
76 increase EC incidence by up to 100% by 2025, relative to the observed incidence in  
77 2005 in some European countries [3]. EC-associated mortality has increased by 15%  
78 with many patients unsuitable for standard surgical treatment due to co-morbidities  
79 and 1 in 4 women experiencing serious surgical complications [4-6]. Current  
80 therapeutics also fail to treat late stage disease and, similar to ovarian cancer, survival  
81 rates are especially poor for advanced EC [7]. Despite all of the above, compared with  
82 other hormonally driven malignancies such as breast or ovarian cancer, EC is a  
83 relatively under-researched area. Further research, therefore, is urgently needed to  
84 formulate effective preventative and treatment modalities.

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

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

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

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

## 110 **Materials and Methods**

111 Extended methods can be found in the supplementary methods section.

### 112 **Cell lines:**

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

## 118 **STR Profile Analysis**

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

## 123 **Cell Culture**

124 All reagents were supplied by Sigma, Dorset, UK unless otherwise stated, cell lines  
125 were maintained in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine  
126 serum (FBS, Biosera, UK), L-glutamine and penicillin/streptomycin benzyl penicillin at  
127 37°C in a 5% (v/v) CO<sub>2</sub> atmosphere and details of cell line maintenance and  
128 modifications relevant to different assays are detailed in the supplementary methods  
129 section.

## 130 **Immunostaining**

131 Immunocytochemistry: Cells were grown in 8-well glass slide chambers until at least  
132 60% confluent, washed with PBS and fixed with 10% (v/v) Neutral Buffered Formalin  
133 (NBF) and permeabilized with 0.2% (v/v) Triton X-100 (BD Biosciences, Oxford, UK)  
134 in PBS. 3 µm-thick paraffin sections of tumour samples grown on CAM were prepared  
135 for immunostaining as previously described [14].

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



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

### 145 **RNA Extraction and Real Time-qPCR**

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

### 155 **Protein extraction and SDS-PAGE**

156 Protein lysates were prepared, quantified and analysed by SDS-PAGE as described  
157 previously [15] for the phosphorylated antibodies. For total ER $\alpha$  blots, samples were  
158 sonicated, and lysates were diluted in x5 Lammeli buffer, electrophoresed through  
159 10% (w/v) polyacrylamide gels. Primary antibodies used were phospho-estrogen  
160 receptor  $\alpha$  (ER $\alpha$  antibody sampler kit (#9024, Cell Signalling, MA), anti-ER $\alpha$  (Abcam)  
161 validated against tubulin (Sigma: for ER $\alpha$ ) or anti-glyceraldehyde 3-phosphate  
162 dehydrogenase (GAPDH). by densitometry using ImageJ [17]

### 163 **Assay for Cell Viability and Proliferation**

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

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

### 168 **Scratch Migration Assay**

169 Linear scratch was made through each confluent monolayer (after overnight  
170 attachment) in 6- well plates, and perpendicular guidelines were drawn on the  
171 underside of the plate intersecting the scratch at 3 points per well. The wells were  
172 washed with PBS after the scratch, to remove any suspended cells, then medium  
173 was gently reapplied. Images were taken at each guideline at 0h, 4h, 6h, 24h and  
174 48h and analysed using MiToBio software plugin [19] for ImageJ [17].

### 175 **Chorioallantoic Membrane Model**

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

### 185 **Statistical Analysis**

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

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

## 191 **Results**

### 192 **Selection of the appropriate cell line(s)**

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

### 202 **Authenticating cell lines**

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

## 211 **Characterisation of the cell lines;**

### 212 **Morphology**

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

### 221 **Designing the experiment**

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

### 228 **Target Modulation**

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

234 **Hormone Regulation**

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

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

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

### 262 ***Chemosensitivity***

263 Doxorubicin is a chemotherapy drug used to treat many types of cancer. The cytotoxic  
264 effect of doxorubicin on ISK and RL95-2 cells was examined to ascertain, the most  
265 suitable concentration to use in the CAM model. The IC<sub>50</sub> for doxorubicin on ISK cells  
266 after 72h treatment as assessed by MTT assay was approximately 0.2 $\mu$ M (Fig. 3A).  
267 Spheroids of ISK in 3D cultures disintegrated with 0.1 $\mu$ M doxorubicin treatment for  
268 seven days (Supplementary Fig. S4) and this was associated with an increase in  
269 active caspase 3 levels (Fig. 3A). The IC<sub>50</sub> for RL95-2 after 96 hours was  
270 approximately 0.03 $\mu$ M. (Fig. 3B). After 72h treatment, no IC<sub>50</sub> was reached for RL95-  
271 2 as assessed by MTT assay. With 1 $\mu$ M Doxorubicin treatment the MTT analysis  
272 showed significant differences between time points, ( $p < 0.0001$ ), FACS analysis also  
273 demonstrated a 60% reduction of the live cell population after 72h treatment with 10 $\mu$ M  
274 of doxorubicin as a secondary method to assess the cytotoxic effect of doxorubicin on  
275 RL95-2. Both assays showed similar effects on viability (Fig. 3B & 3C).

### 276 **Assessing changes functional characteristics**

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

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

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

### 284 **Migratory ability**

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

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

### 302 **Invasion**

303 *In vivo* assessment of invasion

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

## 318 **Discussion**

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



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

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

334 Authentication of the cell lines, using STR profiling ensures that the features of the cell  
335 line have not changed, particularly when they are obtained from other sources than  
336 the reputable, authenticity guaranteed suppliers. We highlight the importance of initial  
337 genomic characterisation by demonstrating a significant genetic drift in the old HEC1A.  
338 This could be due to the cross-contamination with other cell lines, which has become  
339 a prolific problem throughout the world, with an alarming estimation that 15% of cell  
340 lines utilised in the USA to be either misreported or contaminated with other cell lines  
341 [26]. Although the scientific community is slowly combating the problem, caution needs  
342 to be taken when interpreting results from publications that have not authenticated the  
343 cell lines used. In the context of EC research, over the last decade, at least six  
344 publications used the so-called hormone responsive hTERT-EEC cell line, which was  
345 the misidentified breast cancer cell line MCF-7 [27-30]. Although the classical  
346 proliferative response to estrogen is seen in breast and endometrial tissue, the two  
347 tissues have very different responses to some other hormonal agents such as  
348 progestogens and tamoxifen [31, 32]. Therefore, the presumed clinical relevance of *in*  
349 *vitro* studies using hTERT-EEC may not have been completely relevant to the  
350 endometrium. This example highlights the importance of fully understanding the model  
351 being used. Obtaining cell lines from authorised cell banks, which authenticate cell

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

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

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

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

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

400 Many animal models have been employed to assess cancer-associated angiogenesis  
401 and metastasis *in vivo*. Of these, the CAM model has the advantage of being a  
402 relatively cheap, widely available, rapid and high throughput model and although it has  
403 been previously used to assess metastasis and invasion in ovarian cancer cell lines  
404 [35] and benign endometrial tissue [36], we have used this model in the context of EC  
405 cell lines for the first time. Upon inoculating onto the CAM, the EC cell lines would be  
406 severely depleted of growth factors and would not survive long unless the cells invade  
407 into the CAM and establish a vascular supply using the host (chick) vasculature,  
408 therefore the assessment of the cell viability and apoptosis within these newly formed  
409 tumours will reflect the potential aggressiveness of the EC cells. Harvested tumour  
410 established on the CAM can be analysed for markers of proliferation or apoptosis with  
411 IHC or qPCR. We have illustrated that in this model, the well-differentiated ISK cell  
412 line which showed lower invasive features *in vitro*, was unable to produce significant  
413 tumours whereas the less differentiated HEC1A and RL95-2 cells with more invasive  
414 *in vitro* behaviour produced large tumours with an impressive neo-vascularisation.

## 415 **Conclusions**

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

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

## 426 **Acknowledgements**

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432 preparation.

## 433 **Conflict of Interest**

434 No conflict of interests to declare.

435

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539

## 540 **Figure Legends**

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

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

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

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

572 Figure 3. A) Percentage survival of ISK cells treated with Doxorubicin (DOXO)  
573 (0.01,0.1 and 1 $\mu$ M) for 72h in 2D growth, and 3D culture in Matrigel™ treated with  
574 0.1 $\mu$ M DOXO and Vehicle (V) for 72h. IF stained spheroid treated with doxorubicin  
575 shows increased activated caspase 3 (red color counterstained with DAPI). Western  
576 blot showing increased activated caspase 3 in doxorubicin treated cells. . B) Dose  
577 response of RL95-2 cell line to DOXO treatment from 24-96h of treatment with a range  
578 of doses 0.0001-10 $\mu$ M compared to control cells treated with V (DMSO) each time  
579 point using MTT assay. With DOXO treatment at 1 $\mu$ M showed significant differences  
580 between 24h and 96h. C) FACS analysis of 7-AAD and Hoechst 33342 dual stained  
581 RL95-2 cells treated with 1 $\mu$ MDOXO or V (DMSO), showed significant changes in the  
582 percentage of live, necrotic and dead cell populations. Data shown as percentage of  
583 cells detected per quadrant compared to single stained controls. Error bars represent  
584 SEM. Significance was determined using t-test. \*\*\*\*= p<0.0001, \*\*= p <0.005 and \*=  
585 p <0.05.

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

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

601 Figure 5. 3D culture models. A) Timeline for Chorioallantoic Membrane Model (CAM)  
602 assay. E0 indicates day eggs were incubated in the hatchery. At E3 excess albumen  
603 removed using an egg punch and a sterile syringe allowing windows to be cut in the  
604 uppermost side of the egg shell. At E7 CAMs were inoculated with cell lines and  
605 incubated for a further 7 days. For drug or vehicle control treatments Eggs were  
606 injected into the chorioallantoic sac at E11 and E13 with either DMSO (4 $\mu$ L/egg) or  
607 Doxorubicin (3 $\mu$ M) diluted in PBS. At E14 tumours were imaged and excised, fixed in  
608 10% NBF and paraffin wax embedded. B) HEC1A, ISK and RL95-2 grown on CAM.  
609 C) Cell lines grown in Matrigel™ D) IHC of excised tumours of RL95-2 tumours grown  
610 in CAM model sectioned and stained for Ki67, Bax and Bcl-2 after treatment for 72h  
611 with Doxorubicin (DOXO) or DMSO (V) as vehicle control.

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

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616 Supplementary Figure Legends

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

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

632 Supplementary Figure S3.  $17\beta$ -Estradiol (E2) effect on ER $\alpha$  and phospho-ER $\alpha$ . A)  
633 Western blot of phospho-ER $\alpha$  (Ser118) after 72h E2 treatment at a range of  
634 concentrations  $10^{-6}$ - $10^{-9}$ M with a GAPDH control. B) BrdU incorporation with 4h  
635 treatment of  $10^{-8}$ M E2 with 5% charcoal stripped FBS (CSFBS). C) Example images  
636 of BrdU incorporation in response to E2 with DAPI counterstaining. ISK cells grown in  
637 DMEM:F12, phenol-red free media with 5% CSFBS  $5 \times 10^3$  cells seeded per well in 8  
638 well slide chamber with vehicle or E2  $10^{-8}$ M (Sigma) for 2 h. BrdU (5'bromo-  
639 2'deoxyuridine, #B5002, Sigma) added to make  $10\mu$ M concentration. Incubated at

640 37°C for 4 h. Cells washed with PBS and fixed with 10% NBF. Cells denatured with  
641 2M HCl for 1 h, washed in borate buffer and blocked with 2.5% normal horse serum  
642 (Vector labs) for 30 min at room temperature. BrdU incorporation detected using  
643 Mouse monoclonal anti-BrdU antibody 1:200 incubated overnight and followed by  
644 Alexa Fluor 488 secondary antibody incubation. Cells were then washed and mounted  
645 using Vectashield with DAPI (Vector labs). D) Ishikawa cell treated with  $10^{-8}$ M E2 in  
646 media supplemented with 5% Charcoal Stripped FBS assessed for proliferative  
647 response by the MTT assay. Showing no proliferative response to E2 compared with  
648 vehicle (V), EGF used as a positive control. The 5% CSFBS media was not able to  
649 prime the cells to respond to E2 detected by BrdU incorporation or MTT assay.

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

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

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

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

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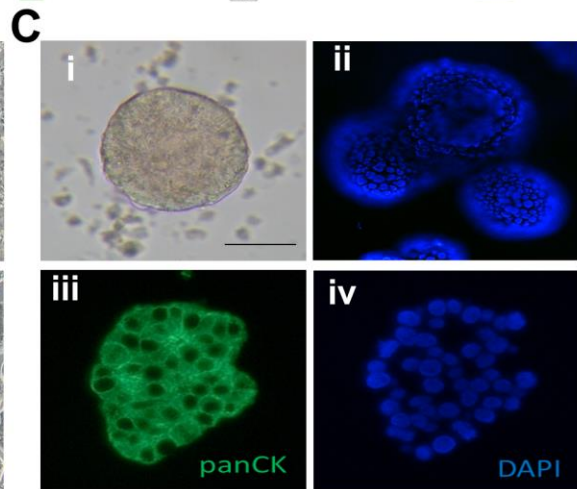
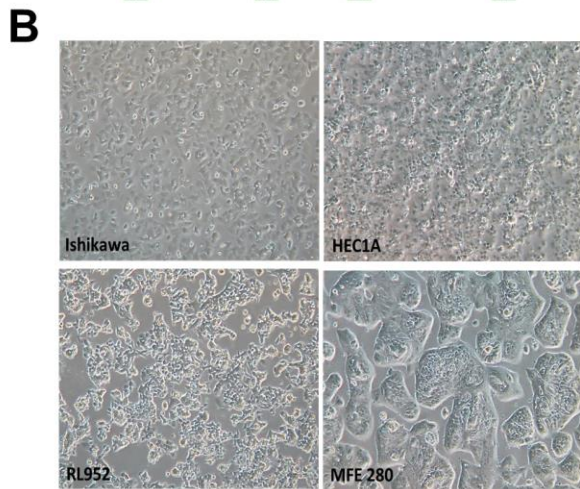
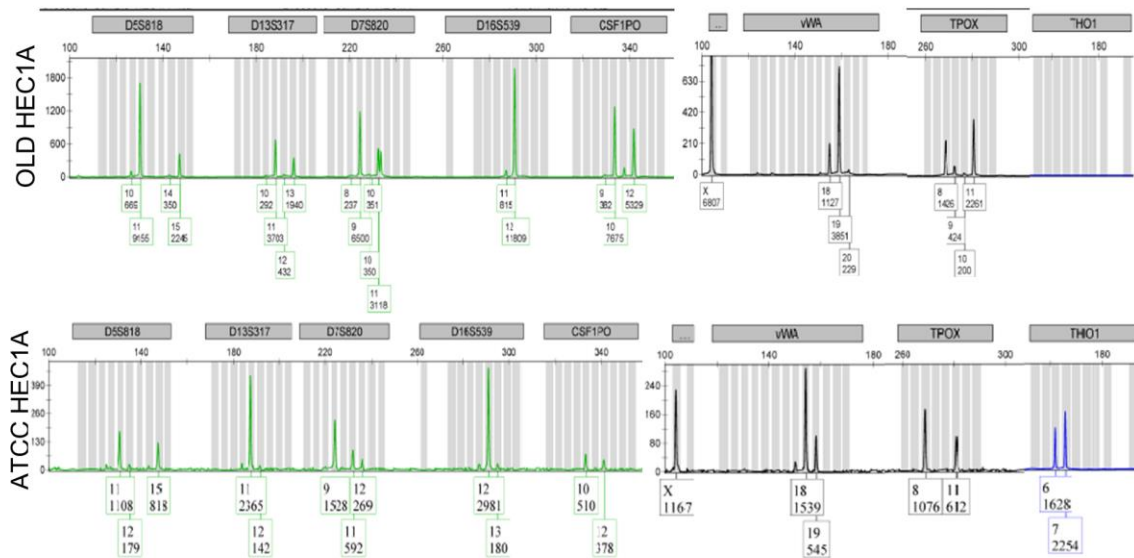
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**A**

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

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



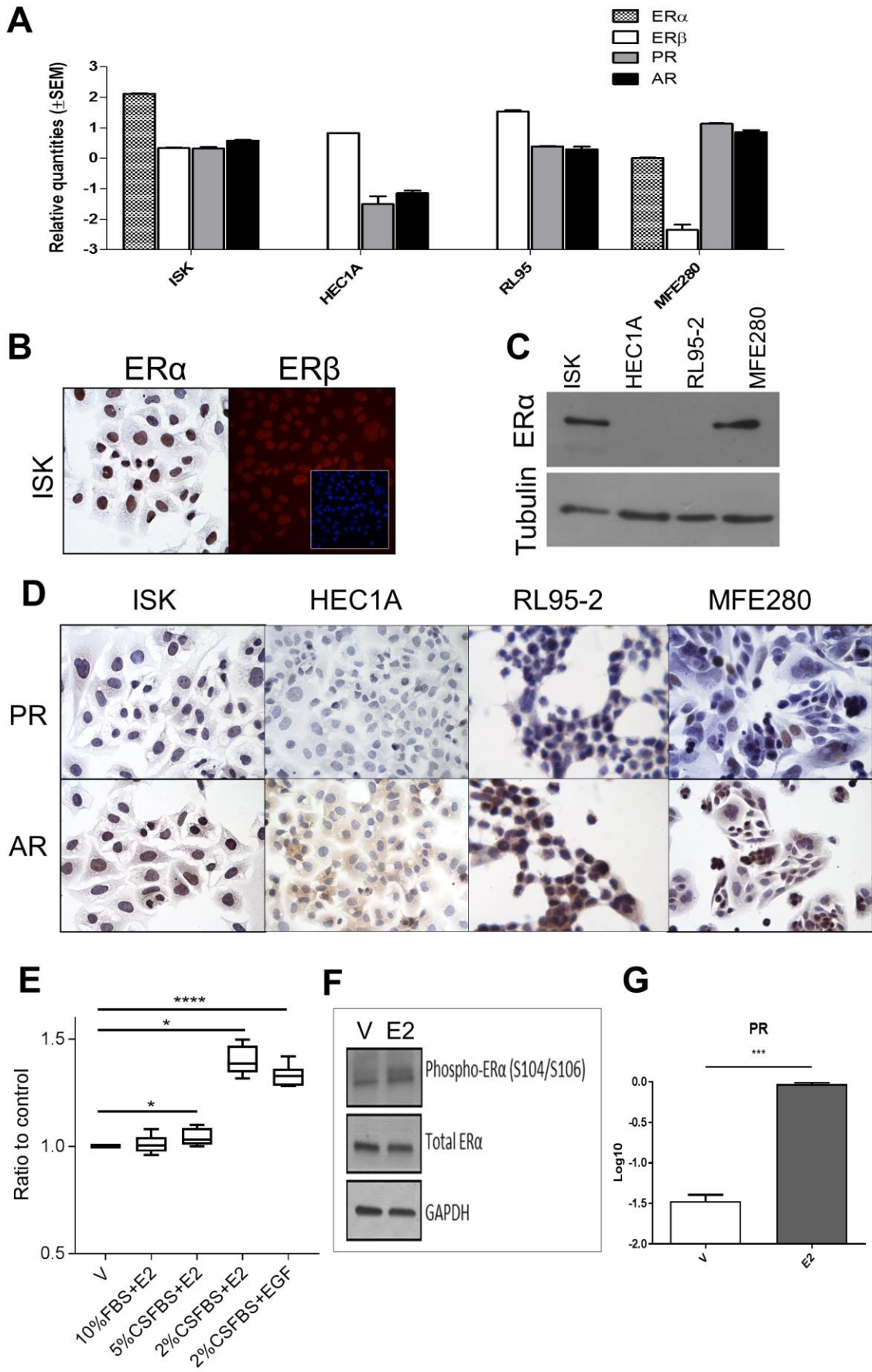
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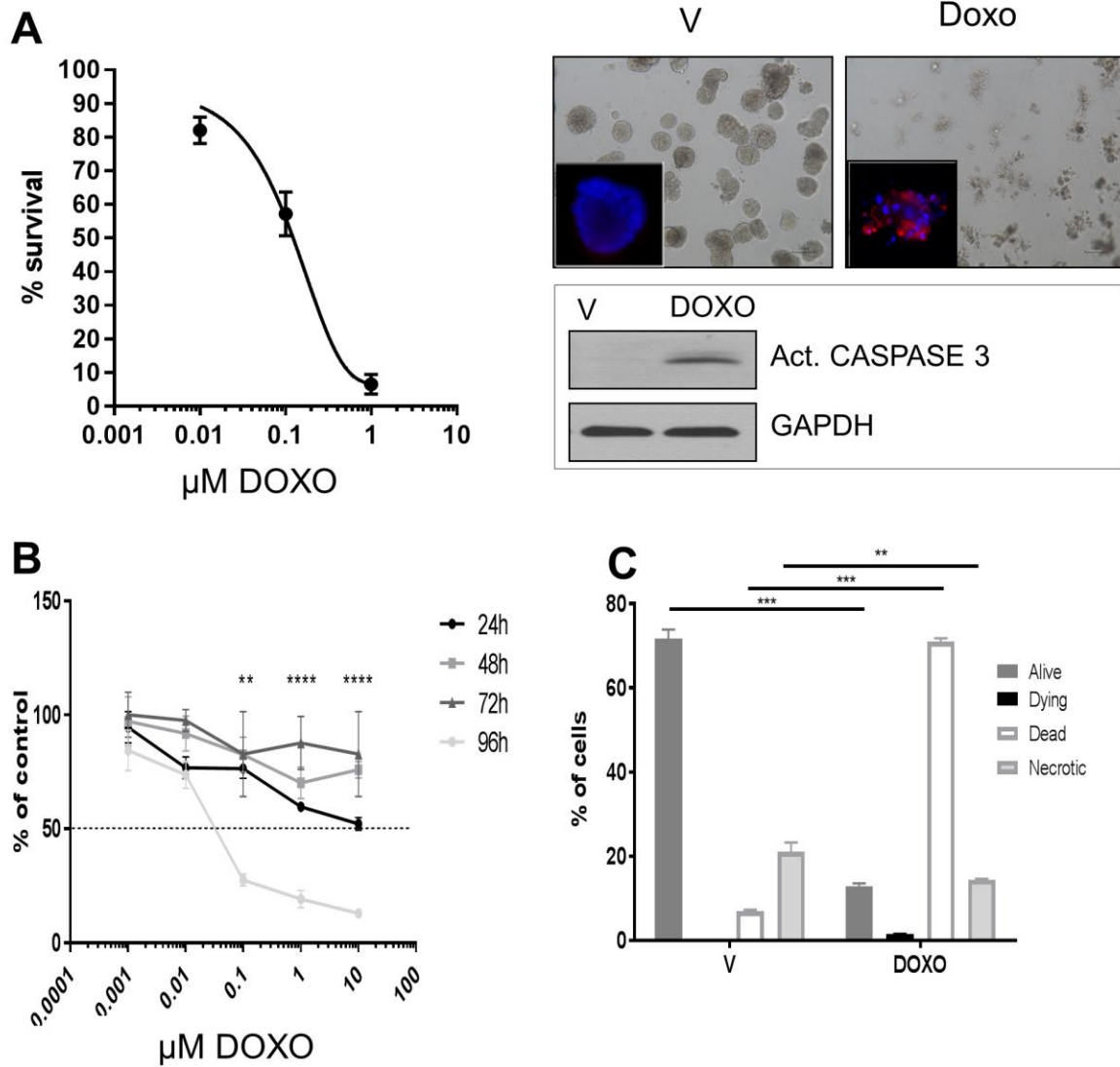
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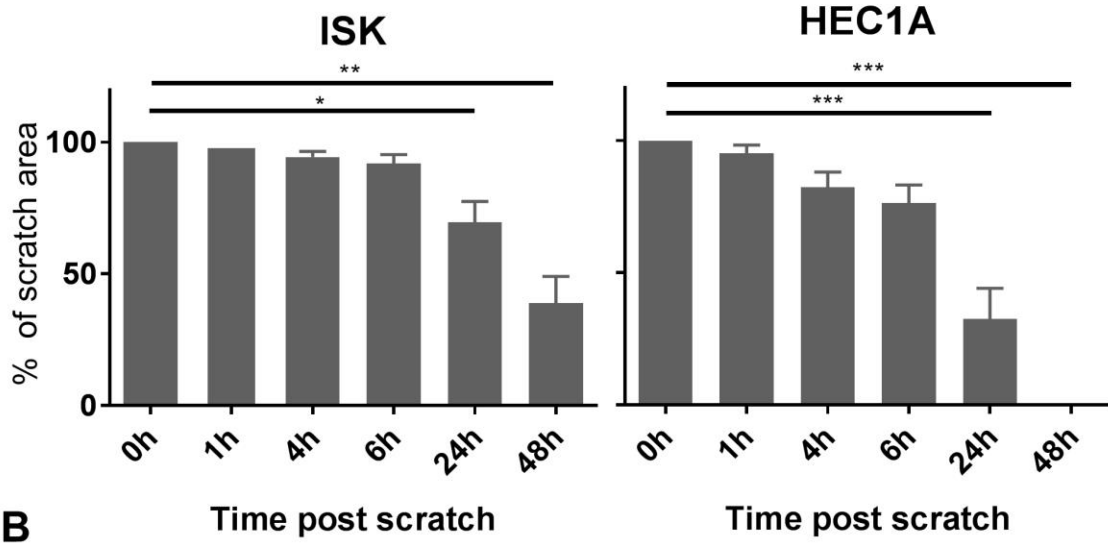
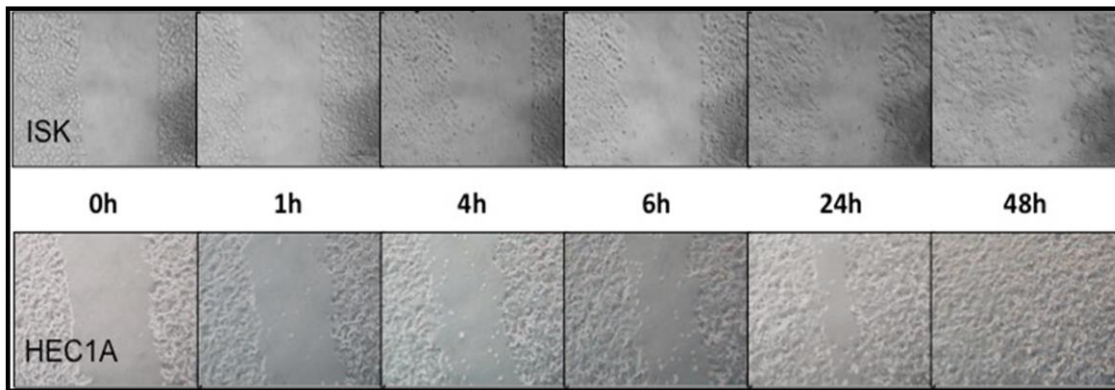
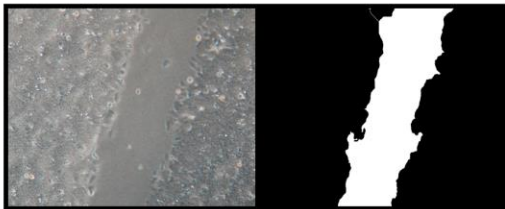
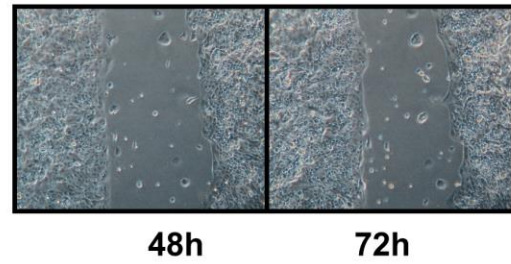
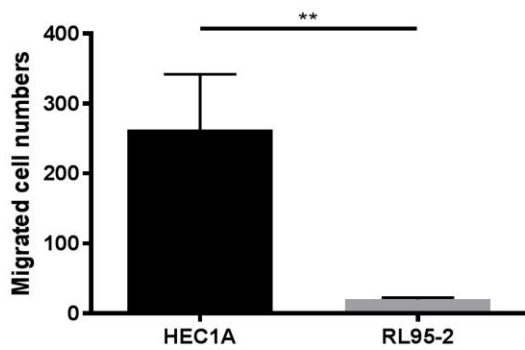
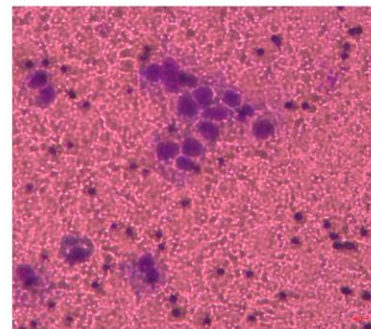
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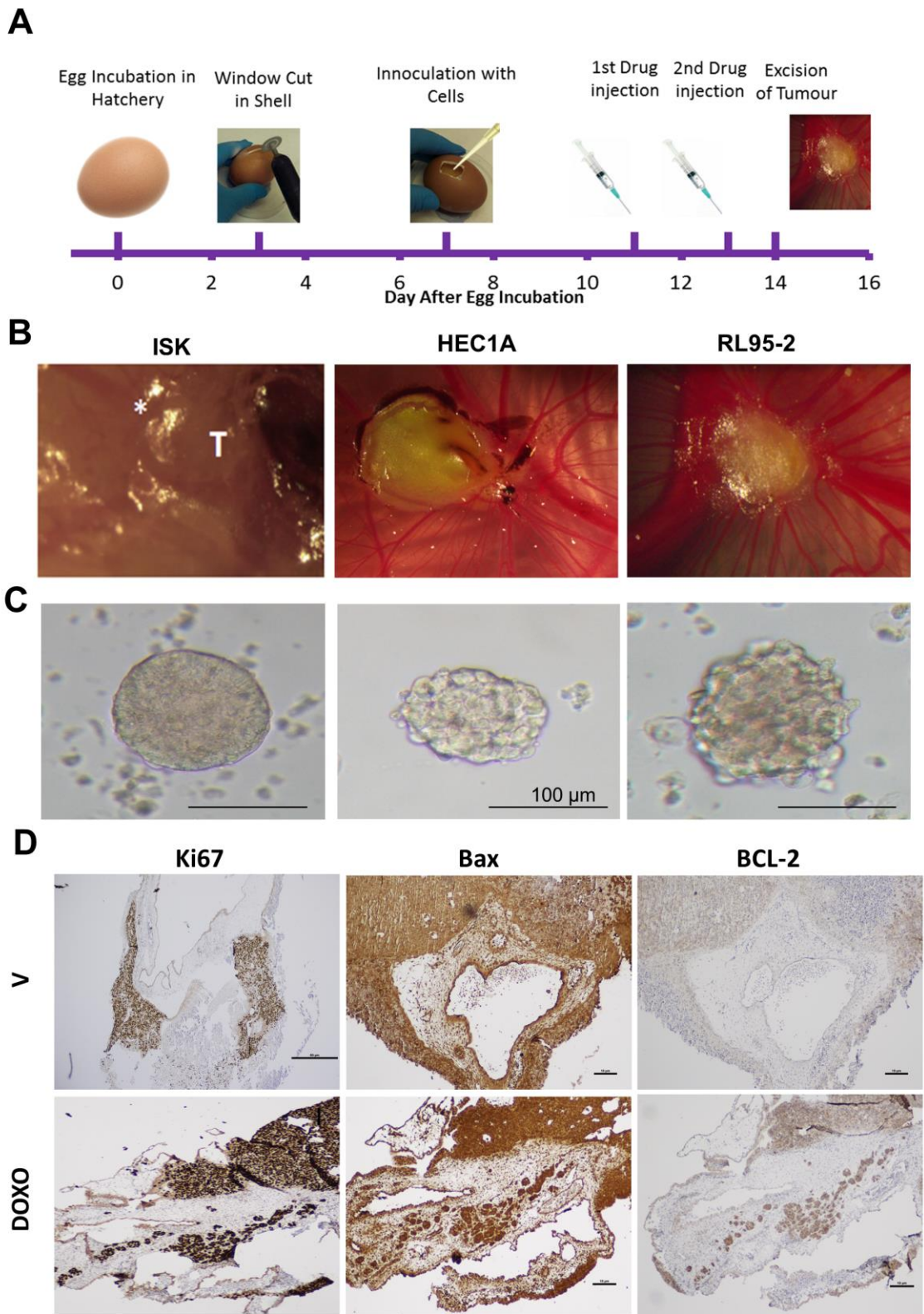
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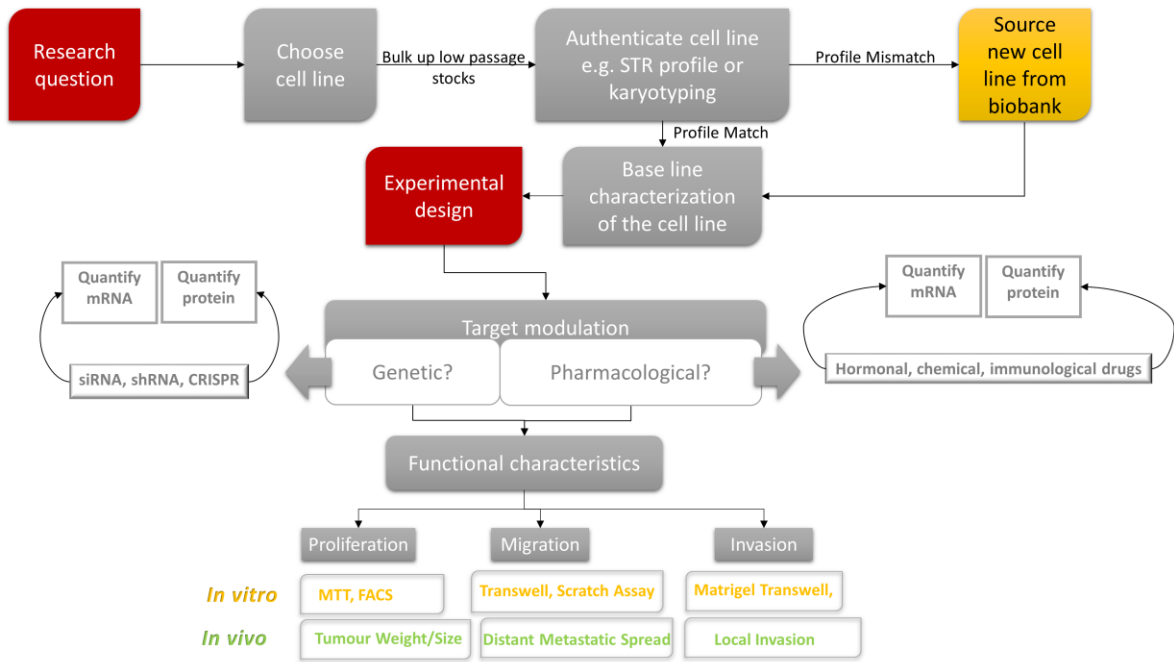
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**A****B****C****D****E****F**



700 Figure 6



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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, epithelioid 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 endometrial
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 <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>
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 endometrioid 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



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	<a href="http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=99040201&amp;collection=ecacc_gc">http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=99040201&amp;collection=ecacc_gc</a>
Ishikawa (Heraklio) O2 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. Epithelial 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 contaminated/ no longer available.
SNG-M	Endometrioid	Japanese	52	IFO50313	JCRB	--	--
TEN	Clear cell carcinoma	Japanese	74	RCB1433	RIKEN	--	Epithelial like morphology.

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723 **Supplementary Table S2.**

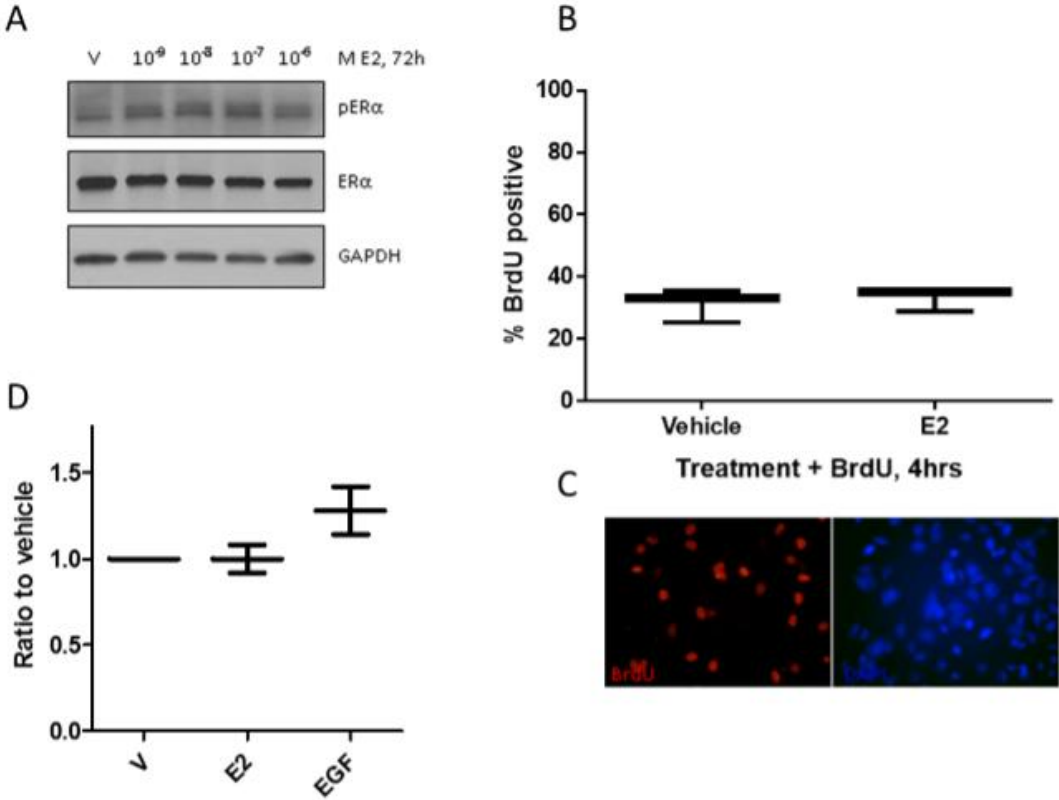
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Lab/Published STR Profile					
Loci	OLD HEC1A	NEW HEC1A	ISK	RL95-2	MFE280
A	X	X	X	X	X
D5S818	11,14,15	11,12,14,15	10,11,12	10,11	11,12
D13S317	11,12,13	11,12	9,12	8,12	10,12
D7S820	9,11	9,11,12	9,10	10	9,10
D16S539	12,13	12,13	9	11,12,13,14	11,12
vWA	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 omitted 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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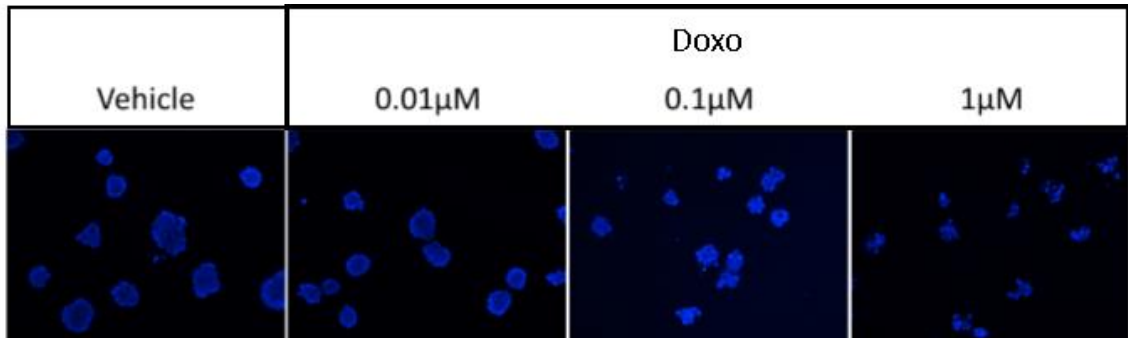
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735 **Supplementary Figure S4.**



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Primer	Sequence	Amplicon	Efficiency	Reference
<b>AR</b>	F: 5'AGGATGCTCTACTTCGCCCC3'	72	99%	Pichler et al., 2013
	R: 5'CTGGCTGTACATCCGGGAC3'			
<b>PR</b>	F: 5'CAGTGGCGTCCAAATGA3'	83	101.3%	Henderson et al., 2003
	R: 5'TGGTGAATCAACTGTATGCTTGA3'			
<b>ER<math>\alpha</math></b>	F: 5'TGATTGGTCTCGTCTGGCG3'	101	92.5%	Henderson et al., 2003
	R: 5'CATGCCCTTACACATTTCCC3'			
<b>ER<math>\beta</math></b>	F: 5'	87	90.3%	BioRad
	R: 5'			
<b>PPIA</b>	5-AGACAAGGTCCCAAAGAC-3	118	96.6%	Jacob et al., 2013
	5-ACCACCCTGACACATAAA-3			
<b>YWHAZ</b>	F: 5'CGTTACTTGGCTGAGGTTGCC3'	69	91.1%	Marullo et al., 2010
	R: 5'GTATGCTTGTGACTGATCGAC3'			

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