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# The impact of 27-hydroxycholesterol on endometrial cancer proliferation

Gibson et al *27-hydroxycholesterol in Endometrial Cancer*

1    **The impact of 27-hydroxycholesterol on endometrial cancer proliferation**

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3    *Running title – 27-hydroxycholesterol in Endometrial Cancer*

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24 **<u>Abstract</u>**

25 Endometrial cancer (EC) is the most common gynaecological malignancy. Obesity is a major

26 risk factor for EC and is associated with elevated cholesterol. 27-Hydroxycholesterol (27HC)

27 is a cholesterol metabolite that functions as an endogenous agonist for Liver X Receptor

28 (LXR) and a selective estrogen receptor modulator (SERM). Exposure to estrogenic ligands

29 increases risk of developing EC however the impact of 27HC on EC is unknown.

30 Samples of stage 1 EC (n=126) were collected from post-menopausal women undergoing

31 hysterectomy. Expression of LXRs (*NR1H3*, LXRα; *NR1H2*, LXRβ) and enzymes required

32 for the synthesis (*CYP27A1*) or breakdown (*CYP7B1*) of 27HC were detected in all grades of

33 EC. Cell lines originating from well-, moderate- and poorly-differentiated endometrial cancers

34 (Ishikawa, RL95, MFE 280 respectively) were used to assess the impact of 27HC or the LXR

35 agonist GW3965 on proliferation or expression of a luciferase reporter gene under the

36 control of LXR- or ER-dependent promoters (LXRE, ERE). Incubation with 27HC or GW3965

37 increased transcription via LXRE in Ishikawa, RL95 and MFE 280 cells (p<0.01). 27HC

38 selectively activated ER-dependent transcription (p<0.001) in Ishikawa cells and promoted

39 proliferation of both Ishikawa and RL95 cells (p<0.001). In MFE 280 cells 27HC did not alter

40 proliferation but selective targeting of LXR with GW3965 significantly reduced cell

41 proliferation (p<0.0001).

42 These novel results suggest that 27HC can contribute to risk of EC by promoting

43 proliferation of endometrial cancer epithelial cells and highlight LXR as a potential

44 therapeutic target in the treatment of advanced disease.

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45 **<u>Introduction</u>**

46 Endometrial cancer (EC) is the most common gynaecological malignancy and the fourth

47 most common cancer in women in developed countries with incidence increasing in line with

48 rising rates of obesity (reviewed in (Onstad, et al. 2016)). Obesity is a major modifiable risk

49 factor for EC and is thought to contribute to increased risk of malignancy in part due to

50 increased exposure to estrogens which enhance risk of aberrant proliferation within the

51 endometrium (Sanderson, et al. 2017). Obesity is also is associated with an adverse

52 metabolic profile which is postulated to independently increase risk of EC (Trabert, et al.

53 2015).

54 A recent meta-analysis supported a positive association between dietary cholesterol

55 consumption and endometrial cancer risk (Gong, et al. 2016). Notably, obesity also puts

56 individuals at risk of developing an adverse, raised, cholesterol profile. Cholesterol

57 metabolites such as the oxysterol 27-hydroxycholesterol (27HC) have been demonstrated to

58 promote cancer growth and metastasis in studies on breast cancer (Nelson, et al. 2013; Wu,

59 et al. 2013a); providing a plausible mechanistic link between increased adiposity and EC

60 risk. 27HC is a primary metabolite of cholesterol, synthesised by the action of sterol 27-

61 hydroxylase (CYP27A1) and metabolised by 25-hydroxycholesterol 7-α-hydroxylase

62 (CYP7B1; [1]). 27HC acts as an endogenous agonist for the Liver X receptor (LXR), a ligand

63 activated transcription factor involved in the regulation of cholesterol homeostasis. Two

64 isoforms of LXR have been identified; LXRα (encoded by *NR1H3)* which is predominantly

65 expressed in the liver, kidney and small intestine but exhibits low expression in other tissues,

66 and LXRβ (encoded by *NR1H2)* which is ubiquitously expressed. Based largely on studies in

67 breast cancer, LXRs have been proposed as a novel anti-cancer target and the LXR-

68 selective agonists GW3965 and T0901317 are reported to decrease proliferation of LXR-

69 expressing breast cancer cell lines (MCF7, T47D, MDA-MB231) as well as the prostate

70 cancer cell line LNCaP (Kim, et al. 2010; Vedin, et al. 2009). To the best of our knowledge

71     LXR expression has not been reported in human EC tissues and the impact of either 27HC

72     or LXR agonists on the endometrium or endometrial malignancies is not known.

73         In addition to activating LXRs, 27HC can also bind estrogen receptors (ER)

74     (Umetani, et al. 2007) and acts as an endogenous selective estrogen receptor modulator

75     (SERM) (DuSell, et al. 2008). 27HC has diverse impacts and its SERM activity is reported to

76     be both tissue-selective and context-dependent. For example, 27HC acts as a competitive

77     antagonist of ERs expressed in the vasculature and can antagonise E2-mediated endothelial

78     cell migration and re-endothelialisation (Umetani et al. 2007). In contrast, in the absence of

79     E2, 27HC is reported to act as an agonist to ERα (ESR1) to increase cell adhesion and

80     expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFA) and

81     interleukin 6 (IL6) (Umetani, et al. 2014) by endothelial cells and macrophages. Notably,

82     27HC is also reported to increase proliferation of ERα-positive breast cancer cell lines and

83     promotes MCF7 tumour xenograft growth in mice by stimulating ER-dependent cell

84     proliferation (Wu et al. 2013a). Given selective LXR agonists have anti-proliferative effects

85     (Vedin et al. 2009), these studies suggest that proliferative effects of 27HC may be mediated

86     via ER and that relative expression of LXR or ER isoforms may define the impact of the

87     ligand.

88         ER isoforms are expressed in EC tissues and ER expression changes with disease

89     progression (Collins, et al. 2009). We have previously reported that ERα is readily detectable

90     in both epithelial and stromal cells in well-differentiated cancers but is significantly reduced in

91     poorly-differentiated cancers. In contrast, expression of ESR2 variants (ERβ1, 2, 5) was

92     readily detected in well, moderate and poorly differentiated stage 1 ECs (Collins et al. 2009).

93     We therefore postulated that 27HC might have distinct effects in EC depending on the

94     bioavailability of ER isoforms present at different stages of disease progression.

95         Obesity and the metabolic syndrome are both associated with an increased risk of

96     developing pre-malignant and malignant endometrial disease (Sanderson et al. 2017) but

97     whether the cholesterol metabolite 27HC has an impact on endometrial cancer

98     risk/progression is not known. In the current study we assessed the expression of the

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99    enzymes required for synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC and

100   assessed expression of the cognate receptors LXRα and LXRβ in primary human stage I

101   endometrial adenocarcinomas (n=126) and postmenopausal endometrial controls (n=9). The

102   impact of 27HC and the LXR-selective agonist GW3965 on ERE- and LXRE-dependent

103   expression of a reporter gene, as well as cellular proliferation, was assessed in three

104   endometrial cancer cell lines which phenocopy well-, moderately- and poorly-differentiated

105   stage I endometrial cancers. Our novel findings demonstrate that 27HC can alter responses

106   in endometrial cancer cells and highlight LXR as a potential therapeutic target. Taken

107   together our findings suggest increased exposure to 27HC may increase risk of development

108   and progression of endometrial cancer.

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109 **Materials and methods**

110 *Human tissue samples*

111 Endometrial adenocarcinoma tissue was collected from post-menopausal women

112 undergoing total abdominal hysterectomy who had been previously diagnosed to have

113 endometrioid adenocarcinoma of the endometrium; they had received no treatment before

114 surgery (supplementary table 1). Written informed consent was obtained from all subjects

115 prior to surgery, and ethical approval was granted by the Lothian Research Ethics

116 Committee (LREC1999/6/4). Methods were carried out in accordance with NHS Lothian

117 Tissue Governance guidelines. All endometrial cancers were confined to the uterus

118 (International Federation of Obstetrics and Gynaecology, FIGO, stage 1 as described in

119 (Collins et al. 2009)). Diagnosis of adenocarcinoma was confirmed histologically by an

120 experienced gynaecological pathologist and tissues were further graded as well

121 differentiated (G1), moderately differentiated (G2) or poorly differentiated (G3). Samples

122 were anonymised and patient follow-up information was not available. However, survival

123 statistics for stage 1 EC in the UK are reported as 99% 1-year survival and 95.3% 5-year

124 survival (Cancer Research UK; http://www.cancerresearchuk.org/health-professional/cancer-

125 statistics/statistics-by-cancer-type/uterine-cancer/ accessed November 2017) and

126 Information Services Division Scotland figures, which cover the stage 1 EC samples

127 collected in the current study, report 92.9% 1-year survival and 83.2% 5-year survival for all

128 uterine cancers (http://www.isdscotland.org/Health-Topics/Cancer/Cancer-Statistics/Female-

129 Genital-Organ/#uterus accessed November 2017).

130 Postmenopausal controls (n=9) were obtained from women undergoing surgery for non-

131 malignant gynaecological conditions. None of the women were receiving hormonal therapy.

132 A total of 126 EC tissue samples were analysed; 3 samples per grade were assessed for

133 immunohistochemistry and n=30 well differentiated cancers, n=64 moderately differentiated

134 and n=32 poorly differentiated samples were assessed for qPCR studies. A minimum of 10

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135    samples at each grade were analysed for each gene, detailed sample numbers are included

136    in supplementary table 2. Tissue for immunohistochemistry was collected in neutral buffered

137    formalin (NBF), RNA extraction samples were collected in RNALater (Qiagen, UK).

138    *Measurement of mRNA*

139    Isolation of mRNAs, preparation of cDNAs, and analysis by qPCR was performed according

140    to standard protocols (Bombail, et al. 2010); samples were quantified by relative standard

141    curve method or by the comparative ΔΔCt method with *CYC* as internal control.

142    Primers/probes are given in supplementary table 3.

143    *Immunohistochemistry*

144    Single antibody immunohistochemistry using 3,3'-diaminobenzidine tetra-hydrochloride

145    (DAB) detection was performed as described previously (Collins et al. 2009). Double

146    immunofluorescence was carried out with antibodies directed against LXR or ERα and the

147    proliferation marker Ki67. Details of antibodies and dilutions are provided in supplementary

148    table 4. Primary antibodies were incubated at 4℃ overnight. Antigen detection was

149    performed using Tyramide signal amplification (Perkin Elmer) system followed by nuclear

150    counterstaining with DAPI (4', 6-Diamidino-2-phenyl-indole dihydrochloride). Negative

151    controls were incubated in the absence of primary antibody but otherwise processed as

152    above; no staining was detected in no primary controls for any of the antibodies used (not

153    shown). Images were captured using a LSM 710 Confocal microscope (Zeiss) at x40

154    magnification.

155    *Cell Cultures*

156    Three endometrial adenocarcinoma cell lines representative of well-, moderately- or poorly-

157    differentiated cancers were used. Ishikawa cells were obtained from the European Collection

158    of Cell Culture (ECACC no 99040201, Wiltshire, UK). This cell line was originally derived

159    from a well-differentiated adenocarcinoma of a 39 year-old woman (Nishida, et al. 1985) and

160  reported to express both ERα and ERβ protein (Johnson, et al. 2007). RL95-2 cells (ATCC®

161  CRL-1671™; hereafter RL95) were originally derived from a Grade 2 moderately

162  differentiated endometrial adenocarcinoma (Way, et al. 1983) and reported to express both

163  ERα and ERβ protein (Li, et al. 2014; Yang, et al. 2008). MFE-280 (ECACC no 98050131)

164  were derived from a recurrent, poorly differentiated, endometrial adenocarcinoma and have

165  low/undetectable expression of ERα and ERβ. Cells were maintained in DMEM/F12 (Sigma)

166  supplemented with 10% FBS, 100U penicillin, streptomycin and 0.25 µg/ml fungizone

167  (Invitrogen, Paisley, UK) at 37°C in 5% $CO_2$. Media for RL95 was supplemented with

168  0.005mg/ml Insulin (Sigma). Cells were incubated with 27-hydroxycholesterol (27HC; Tocris

169  Cat. No. 3907) using stocks diluted in ethanol to give final concentrations ranging from $10^{-5}$M

170  to $10^{-8}$M or GW 3965 hydrochloride (GW; Tocris Cat. No. 2474) using stocks diluted in

171  DMSO to give final concentrations ranging from $10^{-5}$M to $10^{-8}$M. Some cultures were co-

172  incubated with the anti-estrogen fulvestrant (ICI 182,780; Tocris Cat. No. 1047) diluted in

173  DMSO at a final concentration of $10^{-6}$M. Appropriate vehicle control incubations were

174  included in all studies. All cell lines were authenticated using the Promega PowerPlex 21

175  system (Eurofins Genomics, Ebersberg, Germany).

176  *Reporter assays*

177  An adenoviral vector containing a 3xERE-tk-luciferase reporter gene was prepared as

178  described previously (Collins et al. 2009). Cells were cultured in DMEM without phenol red

179  and containing charcoal stripped foetal calf serum (CSFCS) for 24 hours before being

180  infected with Ad-ERE-Luc at a MOI of 25. Activation of LXR-dependent signal transduction

181  was assessed according to manufacturer's instructions using reagents from the Cignal LXR

182  Reporter Kit which includes positive and negative controls as well as a luciferase reporter

183  gene under the control of tandem repeats of the LXR transcriptional response element

184  (LXRE) (Qiagen, CCS-0041L).

185    Cells were treated for 24 hours and luciferase activities were determined using 'Bright Glo'

186    reagents (Promega). Luminescence was measured using Fluostar Microplate Reader (BMG

187    labtech) and fold change in luciferase activity calculated relative to vehicle control for each

188    treatment.

189    *Proliferation assays*

190    The impact of treatments on cell proliferation was assessed using CyQUANT® Direct Cell

191    Proliferation Assay (Thermo Fisher, C35011) according to manufacturer's instructions and

192    nuclear fluorescence measured using Novostar Microplate Reader (BMG labtech). For each

193    cell line investigated, cell number was quantified using a standard curve of known cell

194    numbers and fold change in cell number calculated relative to vehicle control for each

195    treatment.

196    *Statistical analysis*

197    Statistical analysis was performed using Graphpad prism. One-way ANOVA was used to

198    determine significance between treatments in data that were normally distributed. Non-

199    parametric testing was utilised where sample sizes were insufficient to confirm normality of

200    data distribution; Kruskal-Wallis test was used to assess differences between treatments.

201    Where data were analysed as fold-change significance was tested using one sample t test

202    and a theoretical mean of 1. Criterion for significance was $p < 0.05$. All data are presented as

203    mean ± SEM.

204    **Results**

205    *Enzymes that regulate bioavailability of 27-hydroxycholesterol and its cognate receptor LXR*

206    *are expressed in endometrial cancer.*

207    Messenger RNAs encoded by *CYP7B1* and *CYP27A1* were detected in all cancer grades

208    (Figure 1A and B); expression of *CYP7B1* was significantly lower in poorly-differentiated

209    cancers compared to moderately differentiated cancers ($p < 0.05$). Relative expression of

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210    *CYP27A1* tended to be higher in poorly differentiated cancers but this was not significant.

211    We next assessed relative expression of mRNAs encoding the LXR receptors known to bind

212    27HC: *NR1H3* (LXRα) and *NR1H2* (LXRβ) were detected in all cancer grades (Figure 1C

213    and D). Expression of *NR1H3* was significantly lower in moderately-differentiated cancers

214    compared to post-menopausal controls ($p<0.01$). Expression of *NR1H2* did not change

215    between sample groups.

216    *Immunolocalisation of LXR and the proliferation marker Ki67 in endometrial cancer tissue*

217    *sections.*

218    The expression of LXR in endometrial cancer tissue sections was assessed by

219    immunohistochemistry using an antibody that detected both isoforms of LXR (mouse anti-

220    LXR; sc-271064). LXR was readily detected in well-, moderately- or poorly-differentiated

221    cancers and was immunolocalised to both stromal and epithelial cells (supplementary figure

222    1). To assess if LXR expression was associated with cell proliferation within endometrial

223    cancer tissue we performed double immunofluorescence staining for both LXR and the

224    proliferation marker Ki67 (Figure 2). In well-differentiated cancers (Figure 2A), nuclear

225    immunoexpression of Ki67 (red staining) was detected which co-localised (yellow arrows)

226    with LXR expression (green staining, note that single channel views show that the intensity

227    of LXR staining varied between cells). Whilst careful evaluation of single channel views

228    confirmed that the majority of LXR-positive cells were also immunopositive for Ki67 some

229    cells were Ki67-negative (white arrows). In contrast, in moderately-differentiated cancers

230    (Figure 2B) both markers were detected but few cells appeared to co-localise (yellow

231    arrows) although LXR-positive cells (white arrows) were found in close association with

232    proliferating cells. In poorly differentiated cancers (Figure 2C), few cells expressed both

233    markers. Ki67-positive cells were clustered in regions with limited LXR expression and no

234    co-expression of LXR and Ki67 was detected. LXR$^{+}$Ki67$^{-}$ cells (white arrows) were detected

235    close to Ki67$^{+}$ cells. We also assessed the expression of ERα and Ki67 in endometrial

236    cancer tissues (supplementary figure 2) as this receptor is implicated in regulation of

237     proliferation in normal endometrium (Frasor, et al. 2003; Lubahn, et al. 1993). Consistent

238     with our previous study, ERα was not detected in the poorly differentiated cancers (Collins et

239     al. 2009) and immunoexpression of Ki67 was clearly independent of ERα with an increase in

240     abundance of positive nuclei in poor (sample codes 910/2178) as compared to well or

241     moderately differentiated tissue where co-localisation of ERα and Ki67 was readily detected.

242     *27HC activates LXRE- and ERE-dependent transcription in endometrial epithelial cancer*

243     *cells and alters proliferation of endometrial cancer cells*

244     Having demonstrated expression of enzymes and receptors required for 27HC signalling, we

245     extended our observational study by exploring the impact of the ligand on endometrial

246     epithelial cancer cell lines chosen to model well-, moderately- or poorly-differentiated stage I

247     cancers; Ishikawa, RL95 and MFE 280. Protein expression of both LXR isoforms was

248     confirmed by western blot in all cell lines studied (supplementary figure 3A and 3B). We

249     assessed the mRNA expression of LXRs in these cell lines and found that their expression

250     phenocopied that found in tissue samples (Supplementary figure 3). *NR1H3* mRNA

251     expression was significantly decreased in RL95 (moderately-differentiated) cells compared

252     to MFE 280 (poorly-differentiated; $p < 0.01$; Supplementary figure 3C). Consistent with tissue

253     mRNA expression patterns *NR1H2* was not different between cell lines (Supplementary

254     figure 3D). Messenger RNAs encoded by both ER genes; ERα *(ESR1)* and ERβ (*ESR2;*

255     ERβ1 specific primers*)* were detected in all of the cell lines (Supplementary figure 4). *ESR1*

256     mRNAs were significantly reduced in RL95 and MFE280 compared to Ishikawa cells

257     (Supplementary figure 4A) consistent with patterns of expression in intact tissue

258     (supplementary figure 2). *ESR2* mRNA was significantly reduced in MFE280 cells compared

259     to Ishikawa (Supplementary figure 4B). As 27HC is both an endogenous agonist for LXR and

260     a SERM, the impact of 27HC on LXRE- and ERE-dependent transcription was investigated

261     in the endometrial cancer cell lines. 27HC significantly increased LXRE-dependent

262     transcription in a dose-dependent manner in all 3 cell lines and was maximally stimulated by

263     $10^{-5}$M 27HC (Figure 3 A-C). In contrast, 27HC only stimulated ERE-dependent transcription

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264    in Ishikawa cells (Figure 3D) at $10^{-8}$M (p<0.01) and $10^{-7}$M (p<0.0001). The impact of 27HC

265    was abrogated by co-incubation with the anti-estrogen Fulvestrant (ICI 182,780) consistent

266    with ER dependence. In contrast to Ishikawa cells, 27HC had little impact on ERE-

267    dependent transcription in RL95 (Figure 3E) and MFE280 cells (Figure 3F). As 27HC could

268    activate both ERE- and LXRE-promoters, we assessed its impact on cell proliferation (Figure

269    3 G-I). 27HC induced proliferation of Ishikawa cells at concentrations ranging from $10^{-8}$M to

270    $10^{-6}$M (p<0.01) but this was inhibited at the highest concentration ($10^{-5}$M, p<0.0001). 27HC

271    significantly increased proliferation in RL95 cells at concentrations of $10^{-7}$M (p<0.001) or

272    greater. In contrast, 27HC did not alter proliferation of MFE 280 cells at any of the

273    concentrations investigated. Neither RL95 nor MFE280 cell lines expressed *CYP7B1*

274    (supplementary figure 3E and 3F) precluding the potential for in vitro metabolism limiting cell

275    responses to 27HC in these cell lines.

276    *Targeting LXR with the synthetic agonist GW3965 activates LXRE-dependent transcription*

277    *and alters cell proliferation in a cell specific manner.*

278    Incubation of cells with the LXR-selective agonist GW3965 significantly increased LXRE-

279    dependent transcription in a dose-dependent manner (Figure 4) consistent with expression

280    of LXRs in the endometrial cancer cell lines (Supplementary Figure 3). In contrast to 27HC,

281    GW3965   significantly   and   robustly   increased   LXRE-dependent   transcription   at

282    concentrations ≥$10^{-8}$M in Ishikawa (Figure 4A) and RL95 (Figure 4B) and ≥$10^{-7}$M in MFE280

283    cells (Figure 4C). Although LXR reporter responses were similar in the different cell lines,

284    proliferation responses were strikingly different. In Ishikawa cells, treatment with GW3965 at

285    concentrations  $10^{-8}$M  (p<0.01)  and  $10^{-5}$M  (p<0.01)  significantly  increased  proliferation

286    (Figure 4D). In contrast, GW3965 significantly and robustly decreased cell proliferation at all

287    concentrations investigated in both RL95 (Figure 4E) and MFE 280 cells (Figure 4F).

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288    **<u>Discussion</u>**

289    To date, no study has assessed the association between the cholesterol metabolite 27HC

290    and EC. EC incidence rates have increased by ~50% since the early 1990s and

291    approximately 57% of endometrial cancers in the United States have been attributed to

292    being overweight or obese (Cancer Research UK; <u>http://www.cancerresearchuk.org -</u>

293    <u>accessed November 2017,</u> and (Calle and Kaaks 2004)). Although increased exposure to

294    adipose-derived estrogens is believed to increase aberrant proliferation within the

295    endometrium (Zhao, et al. 2016), recent evidence supports an independent role for obesity-

296    associated metabolic factors in modulating EC risk. Notably, both elevated triglycerides and

297    increased dietary cholesterol consumption are reported to be associated with increased EC

298    risk (Gong et al. 2016; Lindemann, et al. 2009). Importantly, concentrations of the

299    cholesterol metabolite 27HC are increased in postmenopausal women (Burkard, et al. 2007)

300    and are associated with increased risk of breast cancer. Several studies have identified that

301    27HC has an adverse impact on breast cancer (Nelson et al. 2013; Wu et al. 2013a) but

302    whether 27HC can affect EC has not previously been investigated.

303    In light of these studies we hypothesised that 27HC signalling could contribute to the

304    aetiology of endometrial cancer and influence disease progression and we investigated this

305    using both archival human tissue as well as cell lines that are derived from different grades

306    of EC. We obtained new evidence for expression of the enzymes required for the both the

307    synthesis (CYP27A1) and breakdown (CYP7B1) of 27HC. As concentrations of *CYP7B1*

308    mRNAs were significantly decreased in poorly- compared to moderately-differentiated

309    cancers and expression of *CYP27A1* did not change significantly across EC grades; we

310    believe this would favour increased bioavailability of 27HC with increasing grade. These

311    findings appear to parallel those reported for ER+ breast cancer where decreased

312    expression of CYP7B1 and increased CYP27A1 has been reported in tumours compared to

313    normal breast tissues (Wu et al. 2013a). Furthermore, we found that the endogenous

314    receptor for 27HC, LXR, was immunolocalised to stage 1 cancers and was expressed

315    throughout the tissue and localised to the nuclei of both stromal and epithelial cells.

316    We sought to establish if 27HC could alter responses in endometrial cancer cells by acting

317    via its cognate receptor, LXR, or via estrogen receptors which are known to regulate

318    endometrial proliferation. 27HC activated LXR-dependent transcription in all cell lines tested.

319    In contrast, we found that 27HC activated ERE-dependent reporter gene expression in well-

320    differentiated cancer cells (Ishikawa; ERα+ERβ+) but not in those from moderately- (RL95;

321    ERα$^{low}$ERβ+) or poorly-differentiated cancers (MFE280; ERα$^{low}$ERβ$^{low}$). However, 27HC

322    increased proliferation of both Ishikawa and RL95 cells but not MFE280 cells consistent with

323    reported ER expression in these cell lines (Johnson et al. 2007; Li et al. 2014; Yang et al.

324    2008). Our immunohistochemistry analysis (supplementary figure 2) supported these in vitro

325    findings. We found that the proliferation marker Ki67 co-localised with ERα in well- and

326    moderately-differentiated cancers consistent with a key role for this receptor in mediating

327    endometrial epithelial cell proliferation (Frasor et al. 2003; Lubahn et al. 1993). In poorly-

328    differentiated cancers, ERα was not detected consistent with previous reports (Collins et al.

329    2009). It has been reported that 27HC, acting as a SERM, can impact on ERα- or ERβ1-

330    dependent regulation of cell function (He and Nelson 2017) and the estrogenic effects of

331    27HC could therefore be mediated via either ER isoform in EC cells. In endometrial

332    endothelial cells, which express ERβ but not ERα, estrogenic effects are mediated via ERβ

333    tethered to Sp1 and not via direct binding to ERE (Greaves, et al. 2013). Furthermore, it has

334    been reported that 27HC promotes proliferation of ERα-positive LNCaP prostate cancer cells

335    via ERβ (Lau, et al. 2000) (Raza, et al. 2017) which may account for the apparent

336    discrepancy between ERE reporter assay and cell proliferation responses in RL95 cells

337    observed in the current study. Taken together, these findings reveal the potential for 27HC

338    generated within the EC tissue microenvironment to influence ER-dependent transcription

339    and proliferation via ERs expressed in early grade stage 1 EC.

340   Although the association between ERs and endometrial proliferation is well recognised,

341   there is limited data investigating the role of LXR in this process. Expression of LXRα and

342   LXRβ mRNA has been previously reported in endometrium and myometrium of mice

343   (Mouzat, et al. 2007) and 27HC is reported to increase mouse uterine weight, consistent with

344   an uterotrophic action, however, whether this was mediated via ER or LXR was not

345   investigated (Wu, et al. 2013b). In mice, targeted ablation of the receptor subtypes revealed

346   that *Lxrα-/-* but not *Lxrβ-/-* females had reduced endometrial areas compared to wildtype

347   mice consistent with a role for LXRα in promoting endometrial growth/proliferation in that

348   species (Mouzat et al. 2007). In the current study we found that LXR co-localised with the

349   proliferation marker Ki67 in well-differentiated but not moderate- or poorly differentiated EC

350   tissues. In vitro assays verified this finding as the synthetic LXR agonist GW3965 had a cell-

351   selective impact on the EC cell lines. In Ishikawa cells GW3965 increased proliferation,

352   whereas in RL95 and MFE280 cells equimolar concentrations of agonist blocked

353   proliferation. Given that LXR expression was detected in all grades of endometrial cancer

354   this may suggest LXR could be an effective therapeutic target in some endometrial cancers,

355   albeit in a grade-dependent context. Indeed, GW3965, is reported to abrogate E2-mediated

356   increases in MCF7 breast cancer cell proliferation and has been proposed as an anti-

357   proliferative ligand in this context (Vedin et al. 2009).

358   LXR classically acts as a heterodimeric partner of retinoid X receptor (RXR). RXR is

359   expressed in the nuclei of endometrial epithelial cells throughout the menstrual cycle

360   (Fukunaka, et al. 2001) as well as in endometrial cancer tissues (Nickkho-Amiry, et al.

361   2012). Interestingly, LXR-RXR functions as a 'permissive' heterodimer and binding of either

362   an LXR agonist or the RXR agonist 9-*cis* retinoic acid activates transcription, while agonism

363   of both dimer partners has an additive effect on activation. Assessment of RXR isoforms in

364   the cell lines used in the current study demonstrated differential expression of RXRs in

365   Ishikawa, RL95 and MFE280 cells which may account for the distinct responses of these cell

366   lines in response to GW3965 treatment (supplementary figure 5). *NR2B1* (RXRα) mRNA

367   expression was greatest in RL95 cells while *NR2B2* (RXRβ) was detected in all cell lines.

368   Notably, mRNA expression of *NR2B3* (RXRγ) was not detected in RL95 cells but was

369   abundant in MFE280 cells. Whether changes in the constitution of the receptor isoforms that

370   contribute to the LXR:RXR heterodimer affect responses requires further investigation,

371   however, previous studies demonstrate that targeting retinoid signalling may affect

372   proliferation of EC cells. Notably, retinoic acid (RA) signalling via retinoic acid receptor

373   (RAR) and RXR is reported to inhibit Ishikawa cell proliferation by inducing cell cycle arrest

374   (Cheng, et al. 2011) and fenretinide, a synthetic derivative of RA, induced apoptosis of

375   Ishikawa cells (Mittal, et al. 2014). These results suggest targeting LXR-dependent signalling

376   with LXR and/or RXR agonists could inhibit proliferation in EC and cancer progression.

377   Changes in the local inflammatory environment that occur during development and

378   progression of endometrial cancer may also increase exposure to 27HC due to infiltration of

379   inflammatory cells. We have previously demonstrated that infiltration of immune cells is

380   increased in endometrial cancer tissues compared to controls. Notably, the numbers of

381   macrophages, neutrophils and dendritic cells were significantly increased in EC tissues

382   (Wallace, et al. 2010) consistent with 27HC-dependent increases in migration of bone

383   marrow-derived CD11b^{+} cells reported in in vitro assays (Raccosta, et al. 2013). In addition,

384   27HC increases secretion of CCL2 from macrophages which enhances recruitment of

385   monocytes (Kim, et al. 2013) and can also upregulate ER-dependent expression of pro-

386   inflammatory genes (Umetani et al. 2014). Notably, as Cyp27a1 is reported to be abundant

387   in macrophages [2], these cells may also contribute to an increase in 27HC within the

388   tumour microenvironment. In support of this idea, increased 27HC concentrations have been

389   reported in breast cancer tumours (Wu et al. 2013b) and increased concentrations of

390   cholesterol have been reported in tumours of various cancer types although they have not

391   been directly measured in EC. 27HC can also promote secretion of TNFA and IL6 from

392   macrophages and TNFA is reported to increase proliferation of human endometrial glandular

393   epithelial cells (Nair, et al. 2013). Thus, although in the current study we only investigated

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394    the direct impact of 27HC on proliferation of endometrial cancer epithelial cells, 27HC may

395    also exacerbate changes within the tissue microenvironment by modulating inflammatory

396    responses and this merits further investigation in animal models.

397    <u>Summary</u>

398    In the current study we provide the first evidence to support a mechanistic link between

399    exposure to elevated cholesterol, biosynthesis of 27HC and EC. Analysis of human stage 1

400    endometrial adenocarcinomas revealed expression of the key metabolising enzymes of

401    27HC was altered in EC consistent with increased exposure to 27HC as EC progresses from

402    well- to poorly-differentiated. Although survival rates for EC are high, incidence rates are

403    increasing in line with rates of obesity and a rising incidence in pre- and peri-menopausal

404    women creates unique therapeutic challenges. Based on our novel findings, we propose that

405    exposure to 27HC may influence disease development/progression by activating ER-

406    dependent pathways to increase epithelial cell proliferation. These results suggest strategies

407    that seek to limit exposure to 27HC through lifestyle modification, lipid-lowering drugs such

408    as statins or novel therapeutics that target 27HC synthesis (CYP27A1 inhibitors) may be

409    effective in reducing endometrial proliferation in women at increased risk of developing EC.

410    Taken together our novel findings suggest that altered cholesterol metabolism, and aberrant

411    exposure to 27HC, may contribute to the development and/or progression of endometrial

412    cancer.

413    <u>Acknowledgements</u>

419    <u>Author contributions</u>

420   Experimental design; DAG, FC & PTKS, experimental procedures; DAG, FC, AEZ, FLC,

421   manuscript preparation; DAG, FC & PTKS.

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520

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1   **Figure legends**

2   **Figure 1. 27HC signalling pathway is expressed in endometrial cancer and altered**

3   **with disease severity.** The expression of *CYP7B1, CYP27A1, NR1H3* (LXRα) and *NR1H2*

4   (LXRβ) relative to internal control gene *CYC* was assessed by qPCR in postmenopausal

5   control endometrium (PM Ctrl) and in endometrial cancer tissue homogenates from well-,

6   moderately- and poorly-differentiated endometrial adenocarcinomas. Relative expression of

7   mRNAs encoding *CYP7B1* (**A**) were decreased in poorly differentiated cancers compared to

8   moderately differentiated cancers but *CYP27A1* was not significantly different (**B**). Relative

9   expression of mRNAs encoding *NR1H3* (**C**; LXRα) were significantly decreased in

10  moderately-differentiated cancers compared to post-menopausal control tissues while

11  *NR1H2* (LXRβ) was not significantly different (**D**). *$p<0.05$, **$p<0.01$. Kruskal-Wallis test with

12  multiple comparisons. PM, n=9; Well n=12-30; Mod, n=42-64; Poor, n=23-32. All data are

13  presented as mean ± SEM.

14  **Figure 2. Expression of LXR and the proliferation marker Ki67 in endometrial cancer.**

15  The expression of LXR (antibody identified both isoforms) and the proliferation marker Ki67

16  was assessed by immunohistochemistry in endometrial cancer tissue sections. In well-

17  differentiated cancers (**A**), LXR was expressed throughout the tissue and localised to the

18  nuclei of both stromal and epithelial cells (green staining). Nuclear immunoexpression of

19  Ki67 (red staining) was detected and co-localised with LXR expression (yellow arrows)

20  although some LXR-positive cells did not co-express Ki67 (white arrows). In moderately-

21  differentiated cancers (**B**) both markers were detected but did not appear to co-localise; only

22  few cells expressed both LXR and Ki67 (yellow arrows). Most LXR-positive cells did not co-

23  express Ki67 (white arrows). This was also true of poorly differentiated cancers (**C**), few cells

24  expressed both LXR and Ki67 (yellow arrows) although LXR-positive cells were found in

25  close association with proliferating cells (white arrows). Images representative of at least 3

26  different patients per cancer grade. Nuclear counterstain DAPI (grey). All scale bars 50µM.

1

27 **Figure 3. 27HC activates LXRE- and ERE-dependent transcription in endometrial**

28 **epithelial cancer cells and alters proliferation.** The cholesterol metabolite 27-

29 hydoxycholesterol (27HC) is the endogenous agonist for LXR and is also classified as

30 selective estrogen receptor modulator. The impact of 27HC on LXRE- (**A-C**) and ERE-

31 dependent (**D-F**) transcription was investigated by luciferase reporter assay in endometrial

32 cancer cell lines; Ishikawa, RL95 and MFE280. 27HC significantly increased LXRE-

33 dependent transcription in a dose-dependent manner in each endometrial cancer cell line.

34 27HC stimulated ERE-dependent transcription only at lower concentrations and was

35 significantly increased by $10^{-8}$M 27HC (p<0.01) and maximally stimulated by $10^{-7}$M 27HC

36 (p<0.0001). The 27HC effect was abrogated by co-incubation with the antiestrgoen

37 Fulvestrant (ICI 182,780; ICI) at all concentrations of 27HC (**D**). 27HC did not increase ERE-

38 dependent transcription in RL95 (**E**) and was only increased by $10^{-5}$M 27HC (p<0.05) in

39 MFE280 cells (**F**). Cell proliferation was assessed by CyQuant® direct proliferation assay in

40 each cell line (**G-I**). Proliferation of Ishikawa cells was increased by $10^{-8}$M (p<0.01), $10^{-7}$M

41 (p<0.01) and $10^{-6}$M (p<0.01) 27HC but decreased by $10^{-5}$M 27HC (p<0.0001; **G**).

42 Proliferation of RL95 cells was increased by $10^{-7}$M (p<0.001), $10^{-6}$M (p<0.01) and $10^{-5}$M

43 (p<0.001) 27HC (**H**). 27HC did not affect proliferation in MFE280 cells (**I**). *p<0.05, **p<0.01,

44 *** p<0.001, **** p<0.0001. One sample t test and a theoretical mean of 1. All data are

45 presented as mean ± SEM.

46 **Figure 4. LXR agonist GW3965 activates LXRE-dependent transcription and alters**

47 **proliferation in endometrial epithelial cancer cells.** The impact of the LXR synthetic

48 agonist GW3965 on LXRE-dependent transcription (**A-C**) and on cell proliferation (**D-F**) was

49 assessed in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. GW3965

50 significantly increased LXRE-dependent transcription in a dose-dependent manner in each

51 endometrial cancer cell line. GW3965 significantly increased LXRE-dependent transcription

52 at all concentrations assessed and was maximally increased by $10^{-6}$M GW3965 in Ishikawa

53 cells (p<0.0001) (**A**) and RL95 cells (p<0.01) (**B**). LXRE-dependent transcription and was not

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54     increased by $10^{-8}$M GW3965 but maximally increased by $10^{-5}$M 27HC (p<0.001) in MFE280

55     cells (**C**). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell

56     line (**D-F**). Proliferation of Ishikawa cells was increased by $10^{-8}$M (p<0.01) and by $10^{-5}$M

57     27HC (p<0.01) (**D**). In contrast, proliferation of RL95 cells was decreased by $10^{-8}$M

58     (p<0.001), $10^{-7}$M (p<0.001), $10^{-6}$M (p<0.01) and $10^{-5}$M (p<0.05) GW3965 (**E**). Proliferation of

59     MFE280 cells was decreased by $10^{-8}$M (p<0.001), $10^{-7}$M (p<0.0001), $10^{-6}$M (p<0.0001) and

60     $10^{-5}$M (p<0.05) GW3965 (**I**). *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001. One sample t

61     test and a theoretical mean of 1. All data are presented as mean ± SEM.
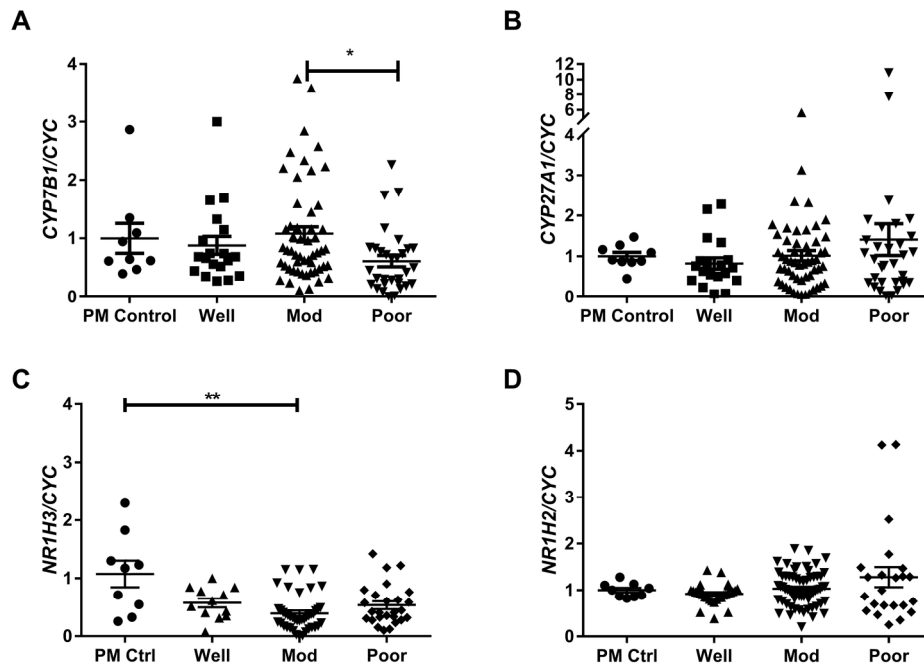
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Figure 1. 27HC signalling pathway is expressed in endometrial cancer and altered with disease severity. The expression of CYP7B1, CYP27A1, NR1H3 (LXRα) and NR1H2 (LXRβ) relative to internal control gene CYC was assessed by qPCR in postmenopausal control endometrium (PM Ctrl) and in endometrial cancer tissue homogenates from well-, moderately- and poorly-differentiated endometrial adenocarcinomas. Relative expression of mRNAs encoding CYP7B1 (A) were decreased in poorly differentiated cancers compared to moderately differentiated cancers but CYP27A1 was not significantly different (B). Relative expression of mRNAs encoding NR1H3 (C; LXRα) were significantly decreased in moderately-differentiated cancers compared to post-menopausal control tissues while NR1H2 (LXRβ) was not significantly different (D). *$p<0.05$, **$p<0.01$. Kruskal-Wallis test with multiple comparisons. PM, n=9; Well n=12-30; Mod, n=42-64; Poor, n=23-32. All data are presented as mean ± SEM.
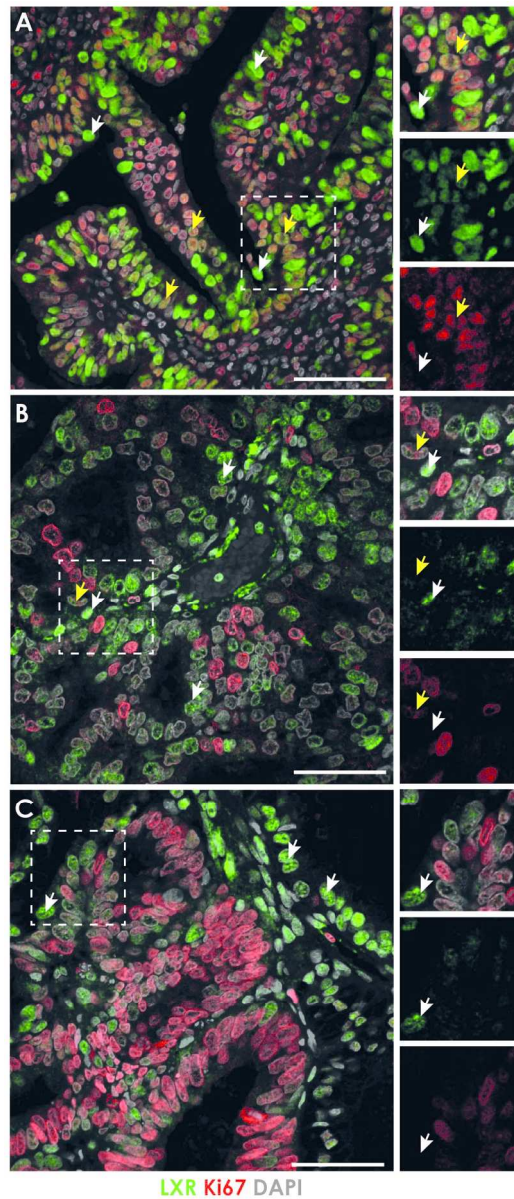
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Figure 2. Expression of LXR and the proliferation marker Ki67 in endometrial cancer. The expression of LXR (antibody identified both isoforms) and the proliferation marker Ki67 was assessed by immunohistochemistry in endometrial cancer tissue sections. In well-differentiated cancers (A), LXR was expressed throughout the tissue and localised to the nuclei of both stromal and epithelial cells (green staining). Nuclear immunoexpression of Ki67 (red staining) was detected and co-localised with LXR expression (yellow arrows) although some LXR-positive cells did not co-express Ki67 (white arrows). In moderately-differentiated cancers (B) both markers were detected but did not appear to co-localise; only few cells expressed both LXR and Ki67 (yellow arrows). Most LXR-positive cells did not co-express Ki67 (white arrows). This was also true of poorly differentiated cancers (C), few cells expressed both LXR and Ki67 (yellow arrows) although LXR-positive cells were found in close association with proliferating cells (white arrows). Images representative of at least 3 different patients per cancer grade. Nuclear counterstain DAPI (grey). All scale bars 50μM.
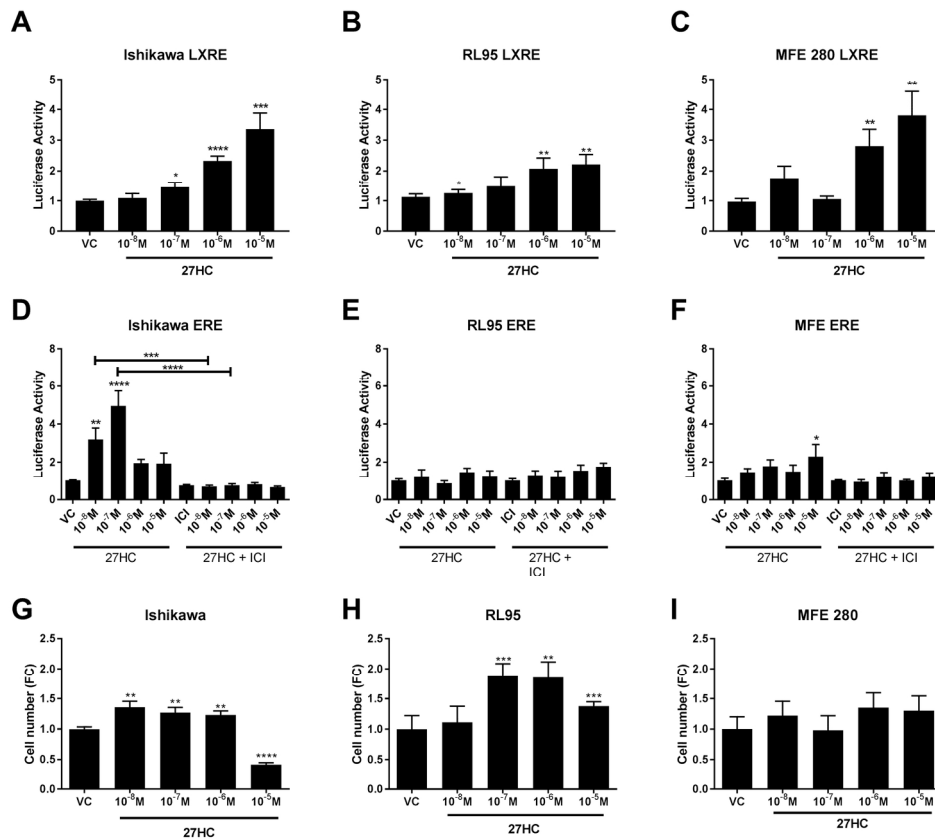
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Figure 3. 27HC activates LXRE- and ERE-dependent transcription in Endometrial epithelial cancer cells and alters proliferation. The cholesterol metabolite 27-hydoxycholesterol (27HC) is the endogenous agonist for LXR and is also classified as selective estrogen receptor modulator. The impact of 27HC on LXRE- (A-C) and ERE-dependent (D-F) transcription was investigated by luciferase reporter assay in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. 27HC significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. 27HC stimulated ERE-dependent transcription only at lower concentrations and was significantly increased by 10-8M 27HC (p<0.01) and maximally stimulated by 10-7M 27HC (p<0.0001). The 27HC effect was abrogated by co-incubation with the antiestrgoen Fulvestrant (ICI 182,780; ICI) at all concentrations of 27HC (D). 27HC did not increase ERE-dependent transcription in RL95 (E) and was only increased by 10-5M 27HC (p<0.05) in MFE280 cells (F). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell line (G-I). Proliferation of Ishikawa cells was increased by 10-8M (p<0.01), 10-7M (p<0.01) and 10-6M (p<0.01) 27HC but decreased by 10-5M 27HC (p<0.0001; G). Proliferation of RL95 cells was increased by 10-7M (p<0.001), 10-6M (p<0.01) and 10-5M (p<0.001) 27HC (H). 27HC did not affect proliferation in MFE280 cells (I). *p<0.05, **p<0.01, *** p<0.001, **** p<0.0001. One sample t test and a theoretical mean of 1. All data are presented as mean ± SEM.
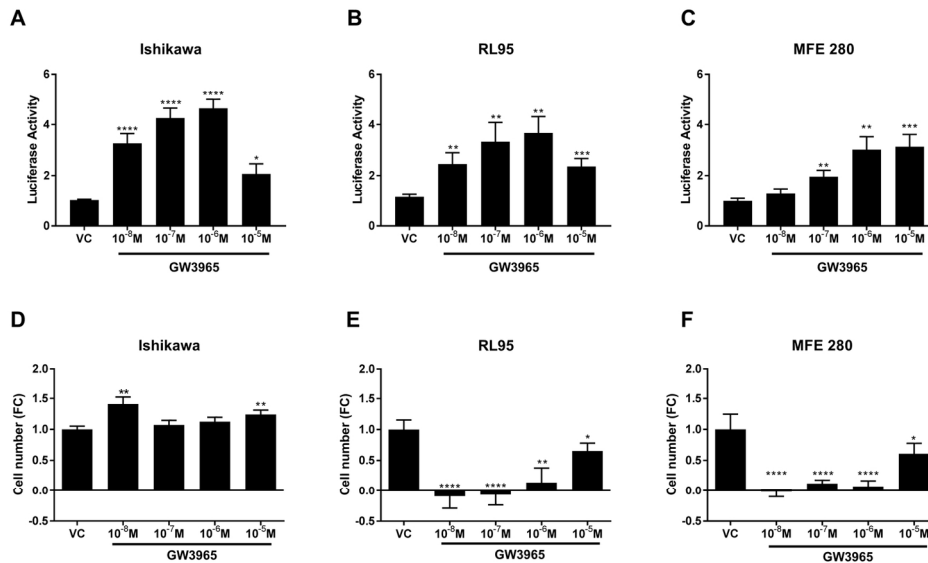
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Figure 4. LXR agonist GW3965 activates LXRE-dependent transcription and alters proliferation in Endometrial epithelial cancer cells. The impact of the LXR synthetic agonist GW3965 on LXRE-dependent transcription (A-C) and on cell proliferation (D-F) was assessed in endometrial cancer cell lines; Ishikawa, RL95 and MFE280. GW3965 significantly increased LXRE-dependent transcription in a dose-dependent manner in each endometrial cancer cell line. GW3965 significantly increased LXRE-dependent transcription at all concentrations assessed and was maximally increased by $10^{-6}$M GW3965 in Ishikawa cells ($p<0.0001$) (A) and RL95 cells ($p<0.01$) (B). LXRE-dependent transcription and was not increased by $10^{-8}$M GW3965 but maximally increased by $10^{-5}$M 27HC ($p<0.001$) in MFE280 cells (C). Cell proliferation was assessed by CyQuant® direct proliferation assay in each cell line (D-F). Proliferation of Ishikawa cells was increased by $10^{-8}$M ($p<0.01$) and by $10^{-5}$M 27HC ($p<0.01$)(D). In contrast, proliferation of RL95 cells was decreased by $10^{-8}$M ($p<0.001$), $10^{-7}$M ($p<0.001$), $10^{-6}$M ($p<0.01$) and $10^{-5}$M ($p<0.05$) GW3965 (E). Proliferation of MFE280 cells was decreased by $10^{-8}$M ($p<0.001$), $10^{-7}$M ($p<0.0001$), $10^{-6}$M ($p<0.0001$) and $10^{-5}$M ($p<0.05$) GW3965 (I). *$p<0.05$, **$p<0.01$, *** $p<0.001$, **** $p<0.0001$. One sample t test and a theoretical mean of 1. All data are presented as mean ± SEM.

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