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Supplementary Information

A nongenomic mechanism for "metalloestrogenic" effects of cadmium in human uterine leiomyoma cells through G protein-coupled estrogen receptor

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Methods

Immunohistochemistry

Human uterine leiomyoma and myometrial tissue samples were embedded in paraffin and stained for GPER by immunohistochemistry. Sections were deparaffinized and rehydrated. Antigen retrieval was performed with proteinase K retrieval (Agilent Technologies, Santa Clara, CA) at room temperature for 2 minutes. Endogenous peroxidase was blocked with IHC/ISH peroxidase block (Enzo Life Sciences, Farmingdale, NY) for 5 minutes. The sections were incubated with Antibody Blocker/Diluent (Enzo Life Sciences, Farmingdale, NY) for 10 minutes, followed by incubation with GPER (Catalog no. ab39742, abcam, Cambridge, MA) polyclonal IgG primary antibody and an equivalent dilution of rabbit IgG, polyclonal – isotype control (negative control; EMD Millipore, Billerica, MA) for 60 minutes at 1:100 dilution. Sections were then incubated with Polyview plus HRP (Enzo Life Sciences, Farmingdale, NY) for 30 minutes. Antigen-antibody complexes were visualized using HDDAB (Enzo Life Sciences, Farmingdale, NY) for 5 minutes and counterstained with ENZO hematoxylin for 30 seconds, dehydrated, cleared, and coverslipped.

Real Time-PCR analysis

Ht-UtLM cells treated with Cd (0.1 μM and 10 μM) were collected at 24 h, 48 h and 72 h for extraction of total RNA using a TRIzol^{TM} Reagent (catalog no. 15596026, Thermo Fisher Scientific). After DNase treatment and RNA purification, one microgram of total RNA was used to prepare cDNA and primed with GPER and GAPDH (house-keeping gene used as a control) and reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (catalog no. 4368814, Thermo Fisher Scientific). The primer sequence for GPER is: forward primer 5'-TGCACCTTCATGTCGCTCTT-3' and reverse primer 5'-GCGGTCGAAGCTCATCCA-3'. The primer sequence for GAPDH is: forward primer 5'-AATCCCATCACCATCTTCCA-3' and reverse primer 5'-TGGACTCCACGACGTACTCA-3'. Real time-PCR was performed with Applied Biosystems Power SYBR Green PCR Mix on an AB cycler (Quantstudio 7 Flex, Thermo Fisher Scientific). The data analysis was based on the ΔΔCt method with normalization to GAPDH.

Table S1. List of antibodies used for western blotting.

Name of antibody	**Dilution**	**Catalog No.**	**Company**
phospho-EGFR (Y845)	1:400	AF3394	R&D
EGFR	1:1000	2232	Cell signaling technology
phospho-p44/42 MAPK	1:1000	9101	Cell signaling technology
p44/42 MAPK	1:1000	9102	Cell signaling technology
phospho-Src	1:1000	2101	Cell signaling technology
Src	1:1000	2123	Cell signaling technology
MMP2	1:1000	4022	Cell signaling technology
MMP9	1:1000	3852	Cell signaling technology
TIMP2	1:1000	5738	Cell signaling technology
TIMP1	1:1000	8946	Cell signaling technology
HB-EGF	1:4000	185555	Abcam
HPRT	1:1000	20975	Santa cruz

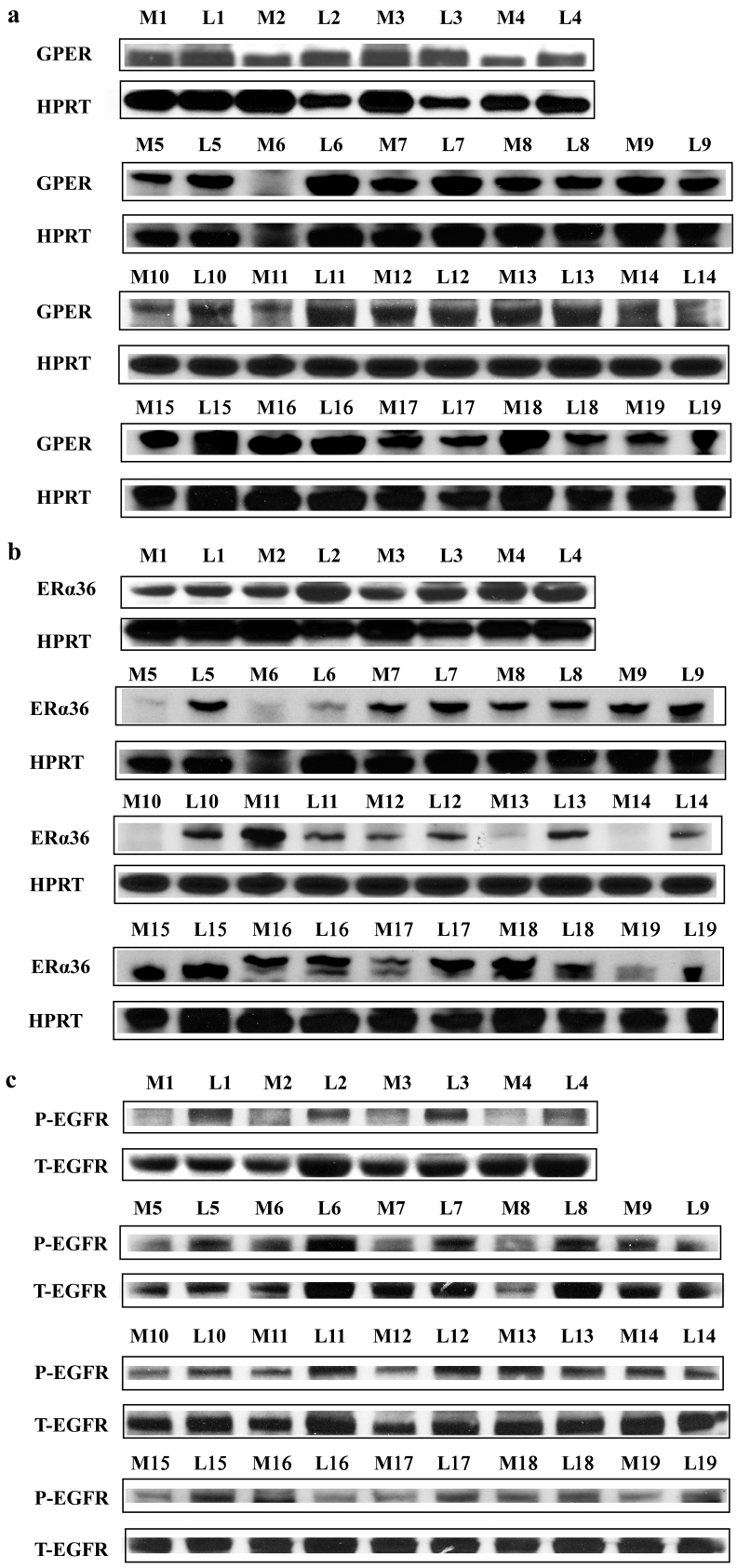
a
M1 L1 M2 L2 M3 L3 M4 L4
GPER
HPRT
M5 L5 M6 L6 M7 L7 M8 L8 M9 L9
GPER
HPRT
M10 L10 M11 L11 M12 L12 M13 L13 M14 L14
GPER
HPRT
M15 L15 M16 L16 M17 L17 M18 L18 M19 L19
GPER
HPRT
b
M1 L1 M2 L2 M3 L3 M4 L4
ERα36
HPRT
M5 L5 M6 L6 M7 L7 M8 L8 M9 L9
ERα36
HPRT
M10 L10 M11 L11 M12 L12 M13 L13 M14 L14
ERα36
HPRT
M15 L15 M16 L16 M17 L17 M18 L18 M19 L19
ERα36
HPRT
c
M1 L1 M2 L2 M3 L3 M4 L4
P-EGFR
T-EGFR
M5 L5 M6 L6 M7 L7 M8 L8 M9 L9
P-EGFR
T-EGFR
M10 L10 M11 L11 M12 L12 M13 L13 M14 L14
P-EGFR
T-EGFR
M15 L15 M16 L16 M17 L17 M18 L18 M19 L19
P-EGFR
T-EGFR

Fig. S1 Expression of GPER, ERα36, and phospho-EGFR (P-EGFR) in patient-matched myometrial (M) and uterine leiomyoma (L) tissues. (a) GPER (b) ERα36 and (c) phospho-EGFR by western blotting. n = 15 pairs of human tissues.

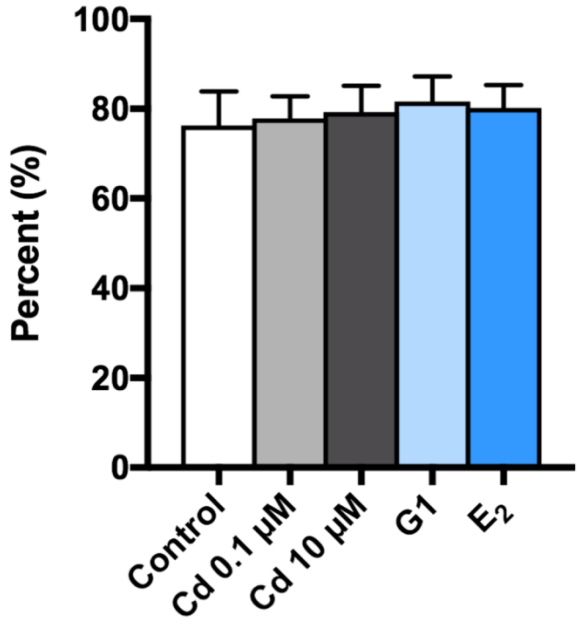

Fig. S2 Percent of ht-UtLM cells positive for GPER by immunofluorescence.

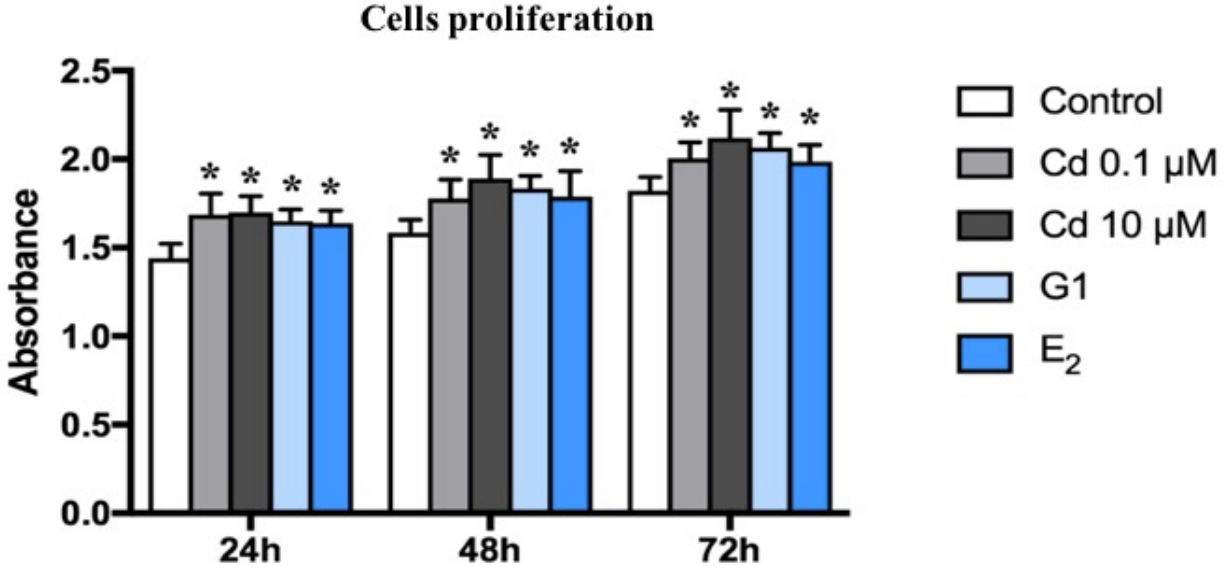

Fig. S3 Effects of Cd on cell proliferation in ht-UtLM cells. *P <0.05 compared to control.

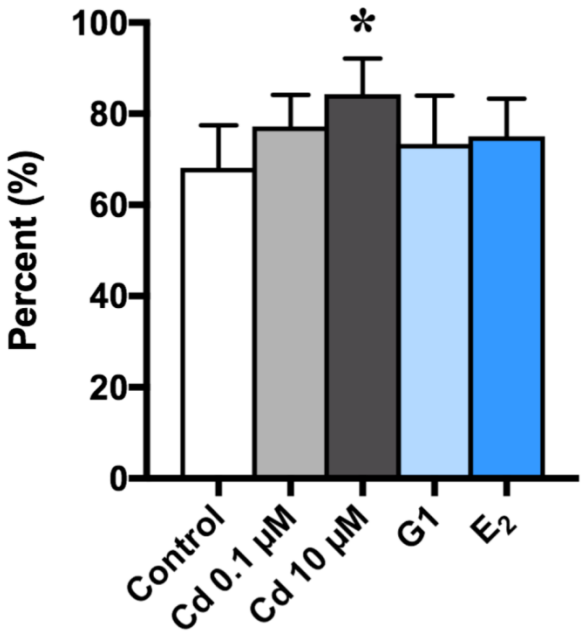

Fig. S4 Percent of ht-UtLM cells positive for phospho-MAPK by immunofluorescence. *P <0.05 compared to control.

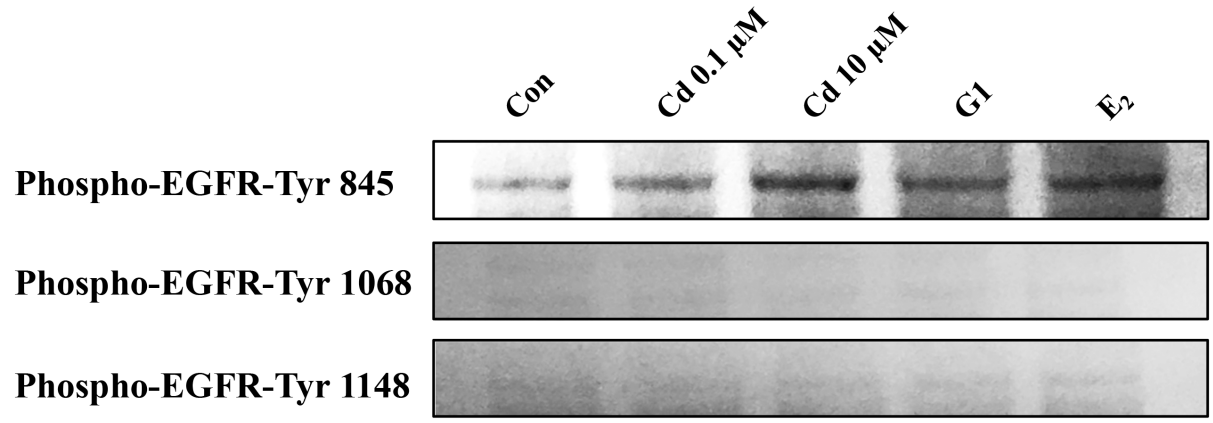

Fig. S5 Activation of different tyrosine (Tyr) residues of EGFR in Cd-treated ht-UtLM cells for 10 min. Representative immunoblots by western blotting.

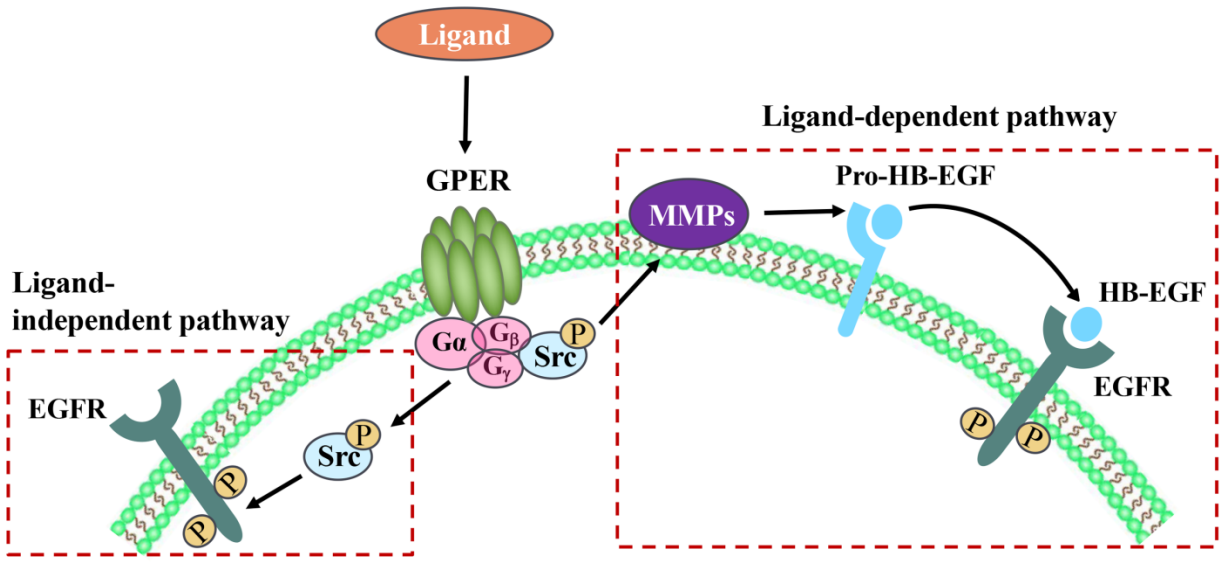

Fig. S6 Mechanisms of EGFR transactivation by GPER.