Epithelial cadherin regulates transition between the naïve and primed pluripotent states in mouse embryonic stem cells

Aseel M. Sharaireh1,2,3 | Lorna M. Fitzpatrick3 | Chris M. Ward1 | Tristan R. McKay3 | Richard D. Unwin2,4

1Division of Dentistry, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK
2Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK
3Stem Cell Group, Centre for Bioscience, Manchester Metropolitan University, Manchester, UK
4Stoller Biomarker Discovery Centre, Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK

Correspondence
Aseel M. Sharaireh, DDS, PhD, Division of Dentistry, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester M13 9NT, UK.
Email: aseel.sharaireh@manchester.ac.uk

Funding information
EU Horizon2020, Grant/Award Number: BATCure (666918); Manchester Academic Health Sciences Centre; Manchester Metropolitan University; University of Jordan, Grant/Award Number: PhD Studentship; University of Manchester

Abstract
Inhibition of E-cad in mouse embryonic stem cells (mESCs) leads to a switch from LIF-BMP to Activin/Nodal-dependent pluripotency, consistent with transition from a naïve to primed pluripotent phenotype. We have used both genetic ablation and specific inhibition of E-cad function in mESCs to assess alterations to phenotype using quantitative mass spectrometry analysis, network models, and functional assays. Proteomic analyses revealed that one third of detected proteins were altered in E-cad null mESCs (Ecad−/− mESCs) compared to wild type (624 proteins were downregulated and 705 were proteins upregulated). Network pathway analysis and subsequent cellular flux assays confirmed a metabolic shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, specifically through mitochondrial complex III downregulation and hypoxia inducible factor 1a target upregulation. Central to this was the transcriptional coactivator EP300. E-cad is a well-known tumor suppressor, its downregulation during cancer initiation and metastasis can be linked to the metabolic switch known as Warburg effect. This study highlights a phenomena found in both primed pluripotent state and cancer stemness and links it to loss of E-cad. Data are available via ProteomeXchange with identifier PXD012679.

KEYWORDS
E-cadherin, iTRAQ, mESCs, metabolic switch, metabolism, pluripotency, proteomics

1 | INTRODUCTION

Pluripotency is classically defined as the cellular capacity to self-renew indefinitely while maintaining the ability to differentiate to cell-types representative of the three embryonic germ layers: ectoderm, endoderm, and mesoderm. Pluripotency in embryogenesis is transient and dynamic; “naïve” stem cells (mESCs) are isolated from the inner cell mass (ICM) of the early blastocyst, maintained in LIF1,2 and dependent on LIF/STAT3 and SMAD1/5/8 signaling networks while “primed” stem cells (mEpiSC) isolated from the later epiblast (E5.5 to E6.5 postimplantation mouse embryos)9,10 rely on SMAD2/3 signaling to maintain pluripotency. Both states are maintained by the well-established pluripotency auto-regulatory transcriptional network of Oct4, Sox2, and Nanog4,10 and defined by a combination of morphological and phenotypic states.11 The transition between states is poorly understood.12 Naive mESCs efficiently form high-quality chimeras following blastocyst injection and implantation whereas primed mEpiSC do not, implying they are developmentally matured.13,14

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

©2020 The Authors. STEM CELLS published by Wiley Periodicals LLC on behalf of AlphaMed Press 2020

Stem Cells. 2020;38:1292-1306. wileyonlinelibrary.com/journal/stem
The transition from naïve to primed pluripotency is known to involve a metabolic switch from oxidative phosphorylation (OXPHOS) to complete reliance on glycolysis.\textsuperscript{15-17} It seems that ATP synthesis in primed cells is uncoupled from mitochondrial respiration through the electron transport chain (ETC), with these cells producing the required energy through Krebs cycle, using lipids and amino acids.\textsuperscript{18} It is suggested that primed cells exploit glycolysis to increase their glycoler backbone to prepare for the lipid biosynthesis necessary to support increased cell division.\textsuperscript{19} However, the initiation and mechanism of this switch in energy production remains unexplored.

The importance of E-cad throughout embryogenesis is well documented.\textsuperscript{12} Primarily E-cad expression segregates the ICM from trophectoderm within the blastocyst and embryos lacking E-cad die during compaction.\textsuperscript{20} Throughout gastrulation E-cad is downregulated with concomitant N-cadherin upregulation; this delaminates epiblast cells from the primitive streak and allows the formation of mesoderm by EMT resulting in neural tube closure.\textsuperscript{21-23} Contrary to this developmental paradigm, mESCs with E-cad depletion/ablation are capable of maintaining pluripotency through activation of Activin/Nodal and FGF2 signaling pathways in preference to LIF/STAT3 and assuming a primed phenotype.\textsuperscript{24,25} This primed mEpiSC pluripotent state has many similarities with an emerging phenotype associated with cancer stem cells (CSCs) such as those developed in in MCF7 mamospheres\textsuperscript{26} which could have profound implications in understanding how CSCs develop during tumorigenesis. Here, we have interrogated the role of E-cad in regulating metabolism of pluripotent mESC by conducting a high-throughput global proteomic quantification combined with metabolic functional assays to characterize the different phenotypes resulting from the genetic or functional inhibition of E-cad. The resultant proteomic and metabolic phenotype was compared to mEpiSC and CSCs. We have applied network modeling and pathway analysis to identify putative novel interactions and proteins that are implicated in this naïve-to-primed metabolic switch. We find that E-cad/EP300 knockouts. Cells were incubated at 37°C and 5% CO\textsubscript{2}. The medium was replenished every 24 hours and the cells were split when at 70% confluence. These cells have been previously characterized by our lab.\textsuperscript{24}

MCF7 (ATCC HTB-22) were cultured in six well tissue culture plates (Corning, Sigma-Aldrich) and supplemented with Eagle’s minimum essential medium (Sigma-Aldrich) with 10% fetal bovine serum (Gibco by Life Technologies). Cells were maintained subconfluent and media was changed every other day.

2.2 Cell lysis

In this study, 75 cm\textsuperscript{2} flasks were used to culture cells to subconfluent cultures. mESCs were washed with phosphate-buffered saline and lysed using 0.5 mL lysis buffer composed of 1 M triethylammonium bicarbonate (TEAB) and 0.1 wt%/vol% sodium dodecyl sulfate (SDS) for 5 minutes. Lysates were collected using cell scrapers and aliquots were frozen at −80°C.

2.3 iTRAQ labeling

The method was performed following the published protocols by Unwin et al.\textsuperscript{27} Protein concentration was determined by Bradford assay (Bio-RAD). Subsequently, 100 μg of protein from each sample was equalized to 55 μL using 1 M TEAB. 0.1 volumes of 50 mM Dithiothreitol (DTT, Fluka) was added to reduce disulphide bonds and incubated at 60°C for an hour. Samples were alkylated by 0.05 volumes of 200 mM iodoacetamide (IAA) at room temperature for 10 minutes. Proteins were digested using 10 μg trypsin (Sequencing Grade Modified Trypsin, Promega) for 100 μg of protein in each sample. Trypsin was reconstituted in 1 M TEAB so that final SDS concentration in the samples is less than 0.05%, trypsin added to samples and incubated at 37°C overnight. After digestion, samples
were dried using a SpeedVac concentrator (Labconco) and frozen at −20°C.

Dried protein digests were resuspended using 1 M TEAB to 30 μL. iTRAQ reagent vials (AB Sciex) were resuspended with 70 μL isopropanol (Fluka) and transferred to a protein digest. Each of the wild-type biological repeat digests was labeled with iTRAQ reagents: 113, 114, and 115, and one technical repeat digest was labeled with 116 reagent. While the three other biological repeats for E-cadherin knockouts were digested and labeled with: 117, 118, and 119 and one technical repeat labeled with 121. All samples were allowed to react at room temperature for 2 hours. Sample volumes were reduced to 30 μL using SpeedVac concentrator to remove isopropanol and stored at −20°C.

2.4 | High pH reverse phase liquid chromatography fractionation

Chromatography buffers have the following compositions: HPLC buffer A: 0.1 vol%/vol% ammonium hydroxide in HPLC water, and buffer B: 0.1 vol%/vol% ammonium hydroxide in Acetonitrile. iTRAQ labeled samples were resuspended in 50 μL of loading buffer (3 vol %/vol% acetonitrile, 0.1 vol%/vol% ammonium hydroxide) and pooled into one glass vial (Chromacol). The final volume was increased to 1800 μL using the same loading buffer. Peptides were fractionated off-line using an Agilent 1200 series LC system loading 900 μL of sample onto a high pH reversed-phase chromatography column (Agilent ZORBAX 300 Extend-C18 4.6 × 150 mm, 3.5 μm) and eluted using a solvent gradient from 5% buffer B and increasing to 30% (buffer B in 35 minutes, and then to 45% buffer B after 1 minute at 45°C and a flow rate of 700 μL/min). Fractions were collected in a 96-well plate, dried using SpeedVac concentrator and stored at −20°C.

2.5 | Low pH reverse phase liquid chromatography and mass spectrometry (MS/MS)

Chromatography buffers have the following compositions: buffer A: 0.1 vol%/vol% formic acid (Sigma) in HPLC, and buffer B: 0.1 vol %/vol% formic acid in acetonitrile. Each dried fraction was resuspended in 27 μL 3 vol%/vol% acetonitrile and 0.1 vol%/vol% trifluoroacetic acid for analysis by low-pH reversed-phase LC using a nanoAcquity LC system (Waters) on-line to a QStar Elite (Q-ToF) mass spectrometer (ABSciex). Injection volume was set to 9 μL. The mass spectrometer is coupled with Electrospray ionizer (Nanospray II, Applied Biosystems-Sciex) and connected to Analyst QS 2.0 software. Peptides were ionized, fragmented, and measured as described previously.27

2.6 | Data analysis

Raw MS data files were uploaded to Protein Pilot analysis software (ProteinPilot version 4.0, AB Sciex) in order to identify and quantify peptides. Proteins were searched against a mouse-specific database (Swiss-Prot Version number 05_2016). Protein and peptide summaries were exported for statistical assessment. Quantified proteins with unused scores equal to or above 1.3 were included, with an overall false discovery rate (FDR) of 0.5%. Log2 ratios were calculated. Statistical significance (wild type vs knockout) was determined using Welch’s t test.

2.7 | Network modeling

Network model analysis was performed using various applications and software to identify hallmark gene sets and the predicted Protein/Protein interactions. These included: gene set enrichment analysis (GSEA),28-30 Reactome,31,32 Cytoscape (Version 3.4.0 downloaded September 2016),33 ModuLand (Operated as in the Szalay-Bekő et al user guide),34,35 minimum connected dominating set (MCDS) (Cytoscape-Plugin Version 1.0),36 GNCPdo (QIAGEN, SABiosciences),37 Venny,38 Agilent Literature Search (3.1.1/LitSearch version 2.69),33 and GraphPad Prism (version 7.03).

2.8 | Cellular flux assay

Metabolic phenotyping was done using Seahorse XFp Analyzer (Seahorse Bioscience; Seahorse XFp Cell Energy Phenotype Test Kit [103275-100]). Around 4000 cells/well were cultured in assay plates the day prior to the assay. Five measures of oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) were taken at basal rates. Further to calculate maximal respiration another five measurements of OCRs were taken after the injection of: 1 μM Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) when testing on mESCs and 0.25 μM when testing on MCF7 (Seahorse Bioscience, 103275-100). Five measurements of ECARs were taken after the injection of 1 μM Oligomycin (Seahorse Bioscience, 103275-100) which is a complex V inhibitor to induce glycolysis. For mitochondrial complex III activity assay 5 μM Antimycin A (Abcam) were used and five measurements for each before and after injection were taken.

2.9 | Reactive oxygen species assay

ROS-ID Superoxide Detection Reagent (Orange) (Enzo Life Sciences) kit was used to measure reactive oxygen species (ROS). Cells were seeded in 96 well-plate (Corning, 3799) at 5000 cells/well the day prior to the assay. Readings were taken using a FLUOstar Omega microplate reader (BMG LABTECH) with standard fluorescein (Ex = 488 nm, Em = 520 nm) and rhodamine (Ex = 550 nm, Em = 610 nm) filter sets. H2O2 levels were measured using the luminescence assay ROS-Glo H2O2 Assay (Promega) using the same plates at the same seeding density. Relative luminescence was recorded using a plate reader (FLUOstar Omega).
FIGURE 1  Outline of results showing culture pictures of wild-type mESCs (WtD3) and E-cadherin knockout mESCs (E-cad−/−-D3). A, Three biological repeats (WtD3-1, WtD3-2, and WtD3-3-1 for the wild type and E-cad−/−-1, E-cad−/−-2, and E-cad−/−-2-1 for the knockout) and one technical repeat (WtD3-3-2 for wild type and E-cad−/−-3-2 for the knockout) were used for each cell line in the relative analysis of the global proteome. Each repeat was labeled with an iTRAQ reagent as indicated by the arrow next to each repeat. B, Immunofluorescence images for WtD3 and E-cad−/− for the three pluripotency markers Oct4, Sox2, and Nanog and for E-cadherin. mESCs, mouse embryonic stem cells.
2.10 | Inhibition of E-cadherin

E-cad protein in WtD3 was inhibited using a peptide (PepA), SWELYYPLRANL, initially generated by Devemy and Blaschuk, and recently shown to act as a reversible suppressor of E-cad signaling. Half a million cells were grown in six well-plate (Corning, 3516) and split every 2 days. A final concentration of 500 nM PepA was used in cell culture medium. The control and the PepA treated cells were passaged once confluent and the media with or without the peptide were replenished every day for 5 days.

2.11 | Fatty acid β-oxidation inhibition

Wt-mESCs, E-cad−/− mESCs and Day 4-PepA-treated cells (PepA-mESCs) were seeded into 96 well-plates at 5000 cells/well. Etomoxir (Sigma) was used at a final concentration of 100 μM and incubated for 24 hours at 37°C and 5% CO2. PepA treatment was continued during these 24 hours so that the PepA treatment is for 5 days. Metabolic activity was measured after incubating the cells with Thiazolyl Blue Tetrazolium Bromide (MTT) and read at 570 nm (FLUOstar Omega).

2.12 | Pyruvate assay

A Pyruvate Assay Kit (Fluorometric) (Abcam, ab65342) was used to determine the concentration of pyruvate in control and experimental samples according to manufacturer instructions.

3 | RESULTS

3.1 | One third of the identified proteome is altered in E-cad knockout

In order to assess the loss of E-cad in mESC maintained in LIF, a labeled global quantitative proteomics approach was used using isobaric tags for relative and absolute quantitation reagents (iTRAQ). Of the 4315 identified proteins, 3896 proteins were quantified (Figure 1A). Ratios were converted to log2 (ratio) and Welch’s t test (unequal variance test) was used to calculate P values for all proteins. A P value of less than .05 was considered significant, resulting in 1329 quantified proteins with statistically significant fold changes. Data were further filtered by mean fold change (KO:WT), where a cutoff of 20% was used when investigating single proteins and a cutoff of 5% was used when formulating pathway analysis. Using the latter, 705 proteins were upregulated and 624 proteins were downregulated. The complete data set can be found in Table S1.

The differentially expressed protein list was searched manually for markers of pluripotency, EMT, differentiation, and putative pathways involved in cellular metabolism. The proteome was also compared to the well characterized distinctive state-specific proteomes and databases (www.stemcellatlas.org).

Based on a 20% FC cutoff (P < .05), data were screened for pluripotency and differentiation markers, as reviewed by Zhao et al. Proteins with the highest and lowest fold change are shown in Tables S1 and S2. Twelve naïve pluripotency markers were shown to be downregulated, seven of which were among the top 40 downregulated proteins with the greatest level of change (DPPA5A, ZFP42, ZFP57, DPPA4, DNMT3L, ESRRB, and TRIM25) and the further five markers (FGF4, UTF1, RIF1, DPPA2, EP300, and GDF3). Primed pluripotency markers LIN28A, OTX2, and GDF3. The Reactome Pathways application through Cytoscape was used to identify the pathways affected by E-cad knockout. The list of changes was uploaded and matched to the pathways in the Reactome database (Figure 2A,B). Major pathways were examined in two ways: the function of the pathways and its end products, and whether the pathway was upregulated or downregulated (Table S3). For the “gene expression” term, several groups of protein were found upregulated. For example, the Eukaryotic Translation Initiation Factors: EIF3G, EIF2S1, EIF3K, EIF3A, EIF4G1, EIF5A, and EIF5 were upregulated. In addition, COP9 signalosome subunits—COPS2-COPs4 and COP8—were found upregulated while esosome complexes—EXOSC2, EXOSC4– EXOSC7, and EXOSC10—were found downregulated. For the “cell cycle” term, all quantified Anaphase-promoting complex proteins (ANAPC1, ANAPC2, and ANAPC7) were downregulated whilst all quantified mini-chromosome maintenance members (MCM2-MCM7) were upregulated. For the “metabolism” term, 8/10 proteins that make up the glycolysis pathway and four proteins of the tricarboxylic acid cycle (TCA) were upregulated. Other proteins that are involved in the metabolism of glucose were found upregulated include LDHA, PCK2, DLAT, PHGDH, DNAJC9, ACAD9, and FASN while ALDH2, GLUD1, GLDC, DHODH, ADH5, BCKDHA, IDH2, G6PDX, FKBP4, and HSD17B10 were found downregulated. In addition, five proteins specific to complex III of the mitochondrial ETC were downregulated in the E-cad−/− mESCs.

GSEA (software.broadinstitute.org/gsea/) was used to compute overlaps with hallmark sets. Hallmark sets are computationally generated, specific, well-defined biological states or processes based on coherent and coordinate expression. All of the statistically
significant search results are shown in Table 1, with criteria set so that FDR value is less than 0.05. Leading the lists were Myc targets, and mTORC1 (mammalian target of rapamycin complex 1) hallmark sets. Interestingly, these cell signaling pathways are regulators of cellular proliferation through their control over cell cycle progression and cellular metabolism, they also strongly possess central roles in cancer progression and proliferation.

3.3 | Putative signaling pathways at the center of the hierarchical layers and EP300 is dominating the network models

We have implemented a network modeling analysis that aims to identify the key regulators of transcription that are central to the protein changes taking place after loss of E-cad. This was achieved through
**TABLE 1** Functional enrichment analysis using GSEA\textsuperscript{28} for the 1329 proteins that has significant P value and differentially expressed (upregulated and downregulated)

| Hallmark set                      | Proteins in overlap | Percent of overlap | P value   | Description                                                                 |
|-----------------------------------|---------------------|--------------------|-----------|----------------------------------------------------------------------------|
| MYC targets                       | 102.00              | 39.53              | 2.21E−72  | A subgroup of genes regulated by MYC                                          |
| MTORC1 signaling                  | 77                  | 38.5               | 8.30E−67  | Genes upregulated through activation of mTORC1 complex                      |
| E2F transcription factors         | 57                  | 28.5               | 2.81E−41  | Genes encoding cell cycle related targets of E2F transcription factors       |
| G2/M checkpoint                   | 44                  | 22                 | 5.38E−27  | Genes involved in the G2/M checkpoint, as in progression through the cell division cycle |
| Oxidative phosphorylation         | 42                  | 21                 | 5.55E−25  | Genes encoding proteins involved in oxidative phosphorylation               |
| Fatty acids metabolism            | 33                  | 20.89              | 7.48E−20  | Genes encoding proteins involved in metabolism of fatty acids               |
| Glycolysis                        | 36                  | 18                 | 2.89E−19  | Genes encoding proteins involved in glycolysis and gluconeogenesis          |
| Unfolded protein response         | 28                  | 24.78              | 3.60E−19  | Genes upregulated during unfolded protein response, a cellular stress response related to the endoplasmic reticulum |
| Xenobiotic metabolism             | 35                  | 17.5               | 2.31E−18  | Genes encoding proteins involved in processing of drugs and other xenobiotics |
| Hypoxia                           | 34                  | 17                 | 1.78E−17  | Genes upregulated in response to low oxygen levels (hypoxia).               |
| Hallmark set for mitotic spindle  | 32                  | 16                 | 9.52E−16  | Genes important for mitotic spindle assembly                                 |
| Upregulated in response to ROS pathway | 17                  | 34.69              | 6.88E−15  | Genes upregulated by reactive oxygen species (ROS)                          |
| DNA repair                        | 25                  | 16.67              | 4.99E−13  | Genes involved in DNA repair                                                |
| Protein secretion pathway         | 20                  | 20.83              | 1.43E−12  | Genes involved in protein secretion pathway                                  |
| Upregulation in response to UV    | 25                  | 15.82              | 1.66E−12  | Genes upregulated in response to UV radiation                               |
| Peroxisome                        | 20                  | 19.23              | 6.85E−12  | Genes encoding components of peroxisome                                     |
| IL2/STAT5 signaling               | 26                  | 13                 | 5.79E−11  | Genes upregulated by STAT5 in response to IL2 stimulation                    |
| Adipogenesis                      | 25                  | 12.5               | 3.14E−10  | Genes upregulated during adipocyte differentiation (adipogenesis)           |
| Apical junction                   | 25                  | 12.5               | 3.14E−10  | Genes encoding components of apical junction complex                         |
| Apoptosis                         | 21                  | 13.04              | 3.72E−09  | Genes mediating programmed cell death (apoptosis) by activation of caspases  |
| HEME metabolism                   | 22                  | 11                 | 3.80E−08  | Genes involved in metabolism of heme (a cofactor consisting of iron and porphyrin) and erythroblast differentiation |
| Androgen response                 | 15                  | 14.85              | 1.09E−07  | Genes defining response to androgens                                        |
| PI3K/Akt/mTOR signaling           | 15                  | 14.29              | 1.84E−07  | Genes upregulated by activation of the PI3K/AKT/mTOR pathway                 |
| Complement system                 | 20                  | 10                 | 7.27E−07  | Genes encoding components of the complement system, which is part of the innate immune system |
| Estrogen response late            | 20                  | 10                 | 7.27E−07  | Genes defining late response to estrogen                                     |
| Interferon gamma response         | 19                  | 9.5                | 2.94E−06  | Genes upregulated in response to IFNG (Gene ID = 3458)                       |
| Bile acid metabolism              | 13                  | 11.61              | 1.25E−05  | Genes involve in metabolism of bile acids and salts                         |
| EMT                               | 17                  | 8.5                | 4.05E−05  | Genes defining epithelial-mesenchymal transition, as in wound healing, fibrosis, and metastasis |
| Estrogen response early           | 16                  | 8                  | 1.38E−04  | Genes defining early response to estrogen                                     |
| P53 pathway                       | 16                  | 8                  | 1.38E−04  | Genes involved in p53 pathways and networks                                  |
| Cholesterol homeostasis           | 9                   | 12.16              | 1.87E−04  | Genes involved in cholesterol homeostasis                                   |
| TGFβ signaling                    | 7                   | 12.96              | 6.50E−04  | Genes upregulated in response to TGFβ1 (Gene ID = 7040)                      |
| Allograft rejection               | 13                  | 6.5                | 3.63E−03  | Genes upregulated during transplant rejection                                |
| Interferon alpha response         | 8                   | 8.25               | 5.19E−03  | Genes upregulated in response to alpha interferon proteins                   |
| Myogenesis                        | 11                  | 5.5                | 2.24E−02  | Genes involved in development of skeletal muscle (myogenesis)                |

Note: The computation resulted in 36 gene sets with statistically significant P value and was run over false discovery rate (FDR) and q value below 0.05. After the analysis was completed, P values were adjusted using Benjamini-Hochberg equations and a P value of less than .03 was considered significant. The analysis gives the number of proteins overlapping the hallmark set of proteins and the number of proteins in the original hallmark set. The number of proteins found matching was used to calculate the percentage of the overlap (% in overlap) based on the number of proteins matching and those provided from the analysis The Hallmark set search is against 50 hallmark sets which condense information from 4000 overlapping protein/gene sets from v4.0 MSigDB collections C1 through C6.\textsuperscript{28}

Abbreviations: EMT, epithelial to mesenchymal transition; GSEA, gene set enrichment analysis; ROS, reactive oxygen species.
modeling protein-protein networks using Cytoscape and its built-in applications that calculate network centrality scores and network hierarchical connectivity. This aimed to provide an estimate of the key regulators mechanistically linked to proteomic changes consequent to E-cad loss. A network model was created to visualize the changes in protein expression because of knocking out E-cad (Figure S1). The network was created based on a BioGRID mouse database and using Cytoscape software.

The list of nodes in Figure 3A are the resultant top modules with 10 core nodes that have the highest assignment value, this identifies PET112L, the modules central node with the highest assigned value, appears to be the key regulator for the list of changes resulted upon losing E-cadherin. The core nodes that appeared more than once in the modules are highlighted with similar colors to show the pattern of repetition.

The list of nodes in Figure 3A are the resultant top modules with 10 core nodes that have the highest assignment value, this identifies PET112L, the modules central node with the highest assigned value, appears to be the key regulator for the list of changes resulted upon losing E-cadherin. The core nodes that appeared more than once in the modules are highlighted with similar colors to show the pattern of repetition.

![Network model analysis formulated based on BioGRID, Agilent Lit Search and Reactome databases and using Cytoscape. A ModuLand application was subsequently applied to investigate the underlying levels of hierarchy that would result in the altered protein expression in the absence of E-cad. All modulated (upregulated and downregulated) proteins and their first neighbors were included in this analysis. ModuLand operated 879 nodes in the module with 395 links. The number of hierarchical level 1 meta-nodes was 43. A, Protein module showing the highest 10 assigned values with 1 as the highest hierarchical module. Each module contains 10 values to formulate a network. PET112L, the modules central node with the highest assigned value, appears to be the key regulator for the list of changes resulted upon losing E-cadherin. The core nodes that appeared more than once in the modules are highlighted with similar colors to show the pattern of repetition. B, The dominating nodes between networks created using two different databases: Reactome Fls and Agilent Literature Search. Agilent Literature Search plugin is a meta-search tool that finds text-based publications from PubMed, OMIM, and USPTO databases and creates interaction networks based on the search result. The second network was imported into Cytoscape using the Reactome Fls database. The up- and downregulated lists of proteins were investigated separately, as the amount of data (1329 protein) was too large for the applications used to build up the networks. Four networks were created using the upregulated protein list and the downregulated protein list. MCDS was used to calculate the Strongest connected components for each network and Venny was used to configure overlaps. MCDS, minimum connected dominating set.](image-url)
the node that has the highest number of undirected connections making it central to the module. A pattern of repetition in the nodes of different modules was noted, for example, the transcriptional regulator HMGA2 that also regulates cell cycle is found in module 1, 6, and 7. Another example is MAPK13, which is an essential component of the MAP kinase signal transduction pathway, is found in module 2, 3, and 5. The highest assigned values and the nodes in the module are thought to be the central regulators of the network.

Furthermore, we created two more networks based on two different databases to eliminate redundancy and to increase the

**FIGURE 4** Legend on next page.
robustness of the analysis. The MCDS plugin was used to approximate the connectors of the directed networks. The plugin runs an algorithm to calculate the largest connected component underlying directed graph and also the strongly connected component (SCC) of the network. SCC in a network cycle is the node that is reached from every other node in that cycle. These mathematical terms have been validated to identify the main drivers and master regulators of gene transcripts in a proteomic network. Here, we implement these algorithms to distinguish the novel key players for governing the E-cad specific regulatory network. MCDS were calculated for all four networks; we assessed the shared nodes between the Agilent Literature search network and the Reactome FIs network for the upregulated and downregulated proteins (Figure 3B). Sixteen SCCs were shared between the networks based on upregulated protein list while downregulated network shared 11 SCCs. Interestingly, the histone acetyltransferase and transcriptional coactivator EP300 was the one node identified as shared between the four resulting networks (two networks based from Reactome and two networks from Agilent literature search). This infers that it is involved in the upregulated protein and the downregulated proteins in the network. This is an attribute that one could only reasonably assign to a transcriptional coactivator/repressor. Ep300 plays many regulatory roles in signaling pathways, mainly pluripotent activation of STAT3 and regulation in hypoxic conditions. Ep300 has previously been found to be downregulated in the proteome upon the loss of E-cad in mESC; however, this was shown to be due to the reduction in nuclear EP300, while the cytoplasmic levels were not affected. Our data indicate that EP300 is acting as a genomic master regulator by shifting its genomic residence to influence gene expression.

3.4 Loss of E-cad switches metabolic behavior to glycolytic through inhibition of complex III in the ETC and decreases ROS

Our pathway analysis highlighted cellular metabolism as the most prominent change with several metabolic proteins differentially expressed due to loss of E-cad. In particular, several complex III subunits were downregulated and several glycolysis enzymes were upregulated (Figure 4A). We sought to confirm a metabolic phenotype by using cellular metabolic flux assays to both WtD3 and E-cad−/− mESCs. Maximal OCRs and ECARs were measured after injection of Oligomycin and FCCP (Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), respectively. E-cad−/− mESCs had significantly lower OCR compared to wild type at both basal and maximal respiration and E-cad−/− mESC, unlike wild type, were unable to respond to ETC uncoupling (Figure 4B [i, iii]). Furthermore, there was a statistically significant increase in the concentration of pyruvate in E-cad−/− mESCs compared to WtD3 indicating a metabolic switch of glycolytic flux from OXPHOS to glycolysis (Figure 4B [iii]). We observed no difference between ECAR levels under steady state or stress conditions (Figure 4B [iv]) but media acidification can be influenced by many factors in such assays. These manifestly different metabolic phenotypes indicate that the loss of E-cad induces a switch from a bivalent energetic state to a more glycolytic state based on modulation of mitochondrial respiratory components.

To functionally evaluate the dependence of complex III function on E-cad expression, we subjected cells to Antimycin A, a complex III inhibitor during a cellular flux assay. OXPHOS was reduced in wild-type cells to the levels of the E-cad−/− mESCs, which showed no significant response to drug (Figure 4B [v]). The relationship between complex III and ROS is well documented; complex III is considered the main producer of ROS. Our pathway analysis identified many proteins that regulate the production of ROS. As well as complex III components, both superoxide dismutase 1 and 2 (SOD1, SOD2) were found downregulated in the E-cad−/− mESCs (Figure 4A). Real-time superoxide production was measured using a fluorescent detection reagent that reacts with oxygen superoxide and H2O2. The superoxide fluorescence in the WtD3 was significantly higher than that in the E-cad−/− mESCs (Figure 4B [vi]), although levels of H2O2 were not statistically different between the two cell lines (Figure S2).

3.5 Exogenous inhibition of E-cad switches mESCs to fatty acid metabolism and induces energy metabolism in cancer stem-cell like cancer cells

To understand whether these identified and characterized changes in the metabolic profiles were due to knockout of E-cad or due to loss of
cell-cell contact a comparative functional approach was carried out. In this approach, a rapid transient inhibition of E-cad was performed using a neutralizing peptide, for 3 and 5 days. The small peptide is known to target the extracellular domain 1 and 2 Histidine-Alanine-Valine (HAV) region of E-cad and consequently disrupts E-cad mediated cell-cell adhesion. After the treatment with the neutralizing peptide (PepA), colonies were deconstructing by day 3 and were separated at day 5 (Figure 5A). Following inhibition for 3 days, ECARs were decreased with no significant change to OCRs (Figures S3A,B). However, after 5 days of E-cad inhibition, both OCRs at basal and maximal conditions were significantly decreased (Figure 5B [i, iii]) and pyruvate concentration was not significantly different in peptide
treated cells (Figure 5B [iii]) while ECARs were decreased at both basal and maximal conditions (Figure 5B [iv, v]).

Interestingly, cell contact independent growth induced by PepA increased the proliferation rate of mESC (Figure 5C [i]) raising the question as to how they source energy when OXPHOS is decreased. To investigate reliance of the mESC lines on this fatty acid oxidation for energy, we used the MTT assay to estimate for the cells’ ability to convert NAD+ to NADH with Etomoxir (a fatty acid β-oxidation inhibitor). The levels of NADH were significantly higher in the PepA-D3 cells compared to WtD3 and E-cad−/− mESCs (Figure 5C [ii]) without Etomoxir. Addition of PepA-D3 to inhibit E-cad-mediated cell-cell contact caused the most significant drop in NADH levels. This indicates the reliance of PepA inhibited mESCs on this pathway for energy production.

We further wanted to investigate if these metabolic changes are found in a breast cancer cell line MCF7 that exhibit a cancer stem cell-like marker expression (CD24-/CD44+) when E-cad is inhibited.49–51 Functional assays revealed that inhibition of E-cad in MCF7 cell line causes a drop in oxygen consumption at basal and maximal conditions (Figure 5D [i, ii]) similar to what was found in the mouse model with no effect on ECAR level at basal condition (Figure 5D [iii]) and slight increase when induced (Figure 5D [iv]). The similar metabolic phenotype observed from E-Cad inhibition in mESC and MCF7 cells is suggestive of a mechanistically similar process defining mESC naive to primed state transition and acquisition of CSCs phenotype in MCF7.

**4 | DISCUSSION AND CONCLUSION**

E-cad preserves LIF/Stat3-dependent naïve pluripotency12 and possesses a central regulatory role to many cellular processes in mESC. In this project, we conducted a quantitative proteomic analysis to compare the proteomic profiles of wild-type mESC with E-cad knockouts. Furthermore, we investigated whether loss of E-cad would affect the energy production in these embryonic cells using two different routes for E-cad inhibition.

Our data show that E-cad depletion alters the proteomic phenotype of the cells and suggests that E-cad occupies modulatory roles in regulating cellular communication, function, and reproduction. Losing E-cad causes alterations beyond cell-cell contact, affecting different gene expression kinetics, bioenergetics, cell signaling, and growth. Many of the protein changes are involved in versatile cellular processes including: signal transduction, RNA replication, and protein production. Extensive network modeling has provided us with significant insights regarding the protein-protein interactions and the key regulators for the set of proteins altered upon E-cad loss.

Importantly, several key naïve pluripotency and differentiation markers were heading the lists of protein changes, acting as a good positive control for our studies. For example, 13 naïve pluripotency markers were downregulated in the E-cad knockout mESC, of which six of the markers have the largest fold-change reduction compared to the other downregulated proteins.

Among the earliest events of transitioning from a naïve to a primed pluripotency is a decrease of the expression of E-cad.9,14 Epithelial adherens junction signaling pathway is found to be strongly associated with the ICM.52 A primed state of pluripotency can be reversed back to naïvety by overexpression of E-cad.53 Moreover, losing E-cad shifts the pathway maintaining pluripotency from a Lif/Stat3 to an Activin/Nodal. This has led to the conclusion that E-cad is essential for the maintenance of naïve pluripotency.12,54 Our new data show that Ep300 is a central regulator of the network resulted from losing E-cad. This transcriptional coactivator alongside CREB-binding protein acetylates Stat3 to stimulate its transactivation activity.38 It has been reported that Stat3 and Ep300 preserve the metabolic phenotype of a naïve state of pluripotency.45,55

Metabolism was the most apparent signature of the E-cad-knockout induced changes in protein expression, whether changes were
investigated through pathway analysis, functional enrichment analysis (GSEA), or through network modeling. Proteins involved in oxidative phosphorylation, glycolysis, amino acid metabolism, and fatty acid metabolism were largely affected. This has translated to functional changes, losing E-cad whether endogenously or exogenously results in switching between different pathways for energy production. Interestingly, there was a difference in the direction of the metabolic switch depending how E-cad was suppressed. There was a switch from a bivalent state of energy production toward a glycolytic state in the E-cad knockout mESC, whereas peptide inhibited mESC appeared to decrease both OXPHOS and glycolysis to use fatty acids. This may be a result in the point of intervention (loss of protein vs inhibition of E-cad activation) or the stable vs transient nature of the E-cad inhibition. More work is required to determine the precise mechanism behind these apparent differences, including longer time courses of peptide-driven E-cad inhibition. However, both models are consistent in that E-cad seems to be central to energy production regulation and regulation of the switch to the primed pluripotency phenotype of energy production.  

The mechanism behind the switch toward glycolysis can also be elucidated based on the number of proteins that serve as Myc targets and mTOR1 targets, the two most significant regulators of our changing proteins based on our unsupervised hallmark analysis. These two pathways have been widely characterized for their roles in regulating growth and metabolism, respectively. A third pathway, Hypoxia Inducible Factor alpha 1 (HIF-1α) is also well known in regulating the switch to glycolysis. Although our data did not quantitify HIF-1α directly, we demonstrated changes in many of the direct and indirect targets of HIF: eight proteins of the glycolysis pathways, mTOR, Ep300, Pyruvate kinases, and pyruvate dehydrogenase kinases. The direct correlation which we describe between losing E-cad and the downregulation of mitochondrial complex III components is an interesting and novel phenomenon, and provides a mechanistic explanation of how cells inhibit OXPHOS during the primed state in embryogenesis concomitant to the downregulation of E-cad. Several groups have suggested that these primed cells decrease flux through the ETC to protect themselves from the oxygen radicals that can subsequently result in the acquisition of damage to their DNA. This is highly relevant to our study where we demonstrated that the downregulation of complex III activity corresponds to a decrease in ROS.

The phenomenon, known as the Warburg effect, is thought to be due to alterations in oncogenes/tumor suppressor expressions. In this study, we provide data that show how E-cad loss can cause a switch similar to that seen in cancer. We also provide a global scheme that highlights how loss of E-cad causes a change in the regulation of 194 c-Myc/ mTOR target proteins and this can have a substantial effect on the tumor suppressor/oncogene homeostasis and the cascade of events that follow.

ACKNOWLEDGMENTS

Special thanks to the University of Jordan for funding this PhD project. We would like to thank the University of Manchester and the Manchester Metropolitan University where all of the work was performed. We would also like to thank the Manchester Academic Health Sciences Centre (MAHSC) for providing the facility for the proteomics work. L. M. F. and T. R. M. are funded by EU Horizon2020 grant BATCure (666918).

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

A.M.S.: conception and design, data analysis and interpretation, manuscript writing; L.M.F.: assembly of data; C.M.W.: conception and design, provision of cell lines and peptides; T.R.M., R.D.U.: conception and design, data analysis and interpretation, financial support, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD012679.

ORCID

Aseel M. Sharaireh https://orcid.org/0000-0003-1242-1315

REFERENCES

1. Nichols J, Smith A. Naive and primed pluripotent states. Cell Stem Cell. 2009;4(6):487-492.
2. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-156.
3. Raz R, Lee C-K, Cannizzaro LA, d’Eustachio P, Levy DE. Essential role of STAT3 for embryonic stem cell pluripotency. Proc Natl Acad Sci USA. 1999;96(6):2846-2851.
4. De Los Angeles A, Ferrari F, Xi R, et al. Hallmarks of pluripotency. Nature. 2015;525(7570):469-478.
5. Kalkan T, Olova N, Roode M, et al. Tracking the embryonic stem cell transition from ground state pluripotency. Development. 2017;144(7):1221-1234.
6. Williams RL, Hilton DJ, Pease S, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature. 1988;336(6200):684-687.
7. Gomes Fernandes M, Dries R, Roost Matthias S, et al. BMP-SMAD signaling regulates lineage priming, but is dispensable for self-renewal in mouse embryonic stem cells. Stem Cell Reports. 2016;6(1):85-94.
8. Brons IG, Smithers LE, Trotter MW, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature. 2007;448(7150):191-195.
9. Tesar PJ, Chenoweth JG, Brook FA, et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature. 2007;448(7150):196-199.
10. Boyer LA, Lee TI, Cole MF, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005;122(6):947-956.
11. Hackett Jamie A, Surani MA. Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell. 2014;15(4):416-430.
12. Pieters T, van Roy F. Role of cell-cell adhesion complexes in embryonic stem cell biology. J Cell Sci. 2014;127(pt 12):2603-2613.
13. Marks H, Kalkan T, Menafra R, et al. The transcriptional and epigenomic foundations of ground state pluripotency. Cell. 2012;149(3):590-604.
14. Weinberger L, Ayyash M, Novershtem N, Hanna JH. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. Nat Rev Mol Cell Biol. 2016;17(3):155-169.
15. Sperber H, Mathieu J, Wang Y, et al. The metabolome regulates the epigenetic landscape during naive to primed human embryonic stem cell transition. Nat Cell Biol. 2015;17(12):1523-1535.

16. Sperber H, Mathieu J, Wang Y, et al. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol. 2015;17(12):1523-1535.

17. Zhou W, Choi M, Margineantu D, et al. HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/HeSC transition. EMBO J. 2012;31(9):2103-2116.

18. Shyh-Chang N, Daley GQ, Cantley LC. Stem cell metabolism in tissue development and aging. Development. 2013;140(12):2535-2547.

19. Folmes Clifford DL, Park S, Terzic A. Lipid metabolism greases the developmental transition events during human embryonic stem cell differentiation. Cancer Res. 2007;67(23):11254-11262.

20. Holms Clifford DL, Park S, Terzic A. Lipid metabolism greases the stem cell engine. Cell Metab. 2013;17(2):153-155.

21. Larue L, Ohsugi M, Hirchenhain J, Kemler R. E-cadherin null mutant embryos fail to form a trophoderm epithelium. Proc Natl Acad Sci USA. 1994;91(17):8263-8267.

22. Butz S, Larue L. Expression of catenins during mouse embryonic development and in adult tissues. Cell Adhes Commun. 1995;3(4):337-352.

23. Eastham AM, Spencer H, Soncin F, et al. Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. Cancer Res. 2007;67(23):11254-11262.

24. Soncin F, Mohamet L, Eckardt D, et al. Abrogation of E-cadherin-mediated cell–cell contact in mouse embryonic stem cells results in reversible LIF-independent self-renewal. Stem Cells. 2009;27(9):2069-2080.

25. Mohamet L, Lea ML, Ward CM. Abrogation of E-cadherin-mediated cellular aggregation allows proliferation of pluripotent mouse embryonic stem cells in shake flask bioreactors. PLoS One. 2010;5(9):e12921.

26. Lamb R, Oszvari B, Bonuccelli G, et al. Dissecting tumor metabolic heterogeneity: telomerase and large cell size metabolically define a sub-population of stem-like, mitochondrial-rich, cancer cells. Oncotarget. 2015;6(2):21992-21905.

27. Unwin RD, Griffiths JR, Whetton AD. Simultaneous analysis of relative protein expression levels across multiple samples using iTRAQ isobaric tags with 2D nano LC-MS/MS. Nat Protoc. 2010;5(9):1574-1582.

28. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The molecular signatures database hallmark gene set collection. Cell Syst. 2015;3(6):417-425.

29. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005;102(43):15545-15550.

30. Mootha VK, Lindgren CM, Eriksson K-F, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 2003;34(3):267-273.

31. Croft D, Mundo AF, Haw R, et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2014;42(Database issue):D472-D477.

32. Fabregat A, Sidirooulos K, Garapati P, et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2016;44(Database issue):D481-D487.

33. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13(11):2498-2504.

34. Szalay-Beko M, Palatoi R, Szappanos B, et al. ModuLand plug-in for cytoscape: determination of hierarchical layers of overlapping network modules and community centrality. Bioinformatics. 2012;28(16):2202-2204.

35. Kovács IA, Palatoi R, Szalay MS, Csermely P. Community landscapes: an integrative approach to determine overlapping network module hierarchy, identify key nodes and predict network dynamics. PLoS One. 2010;5(9):e12528.

36. Nazarief M, Wiese A, Will T, et al. Identification of key player genes in gene regulatory networks. BMC Syst Biol. 2016;10(1):88.

37. Shilov IV, Seymour SL, Patel AA, et al. The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics. 2007;6(9):1638-1655.

38. Wang R, Cherukuri P, Luo J. Activation of Stat3 sequence-specific DNA binding and transcription by p300/CREB-binding protein-mediated acetylation. J Biol Chem. 2005;280(12):11528-11534.

39. Deveny E, Blaschuk OW. Identification of a novel dual E- and N-cadherin antagonist. Peptides. 2009;30(8):1539-1547.

40. Segal JM. The Role of E-Cadherin in Regulating Embryonic Stem Cell Pluripotent States [PhD thesis]. Manchester, UK: University of Manchester; 2015:137-145.

41. Mookerjee SA, Brand MD. Measurement and analysis of extracellular acid production to determine glycolytic rate. J Vis Exp. 2015;106:e53464.

42. Bleier L, Dröse S. Superoxide generation by complex III: from mechanistic rationales to functional consequences. Biochim Biophys Acta. 2013;1827(11):1320-1331.

43. Xu H, Tian Y, Yuan X, et al. The role of CD44 in epithelial-mesenchymal transition and cancer development. Onco Targets Ther. 2015;8:3783-3792.

44. Sun H, Jia J, Wang X, et al. CD44+/CD44- breast cancer cells isolated from MCF-7 cultures exhibit enhanced angiogenic properties. Clin Transl Oncol. 2013;15(1):46-54.

45. Wu Y, Sarkissyan M, Vadgama JV. Epithelial-mesenchymal transition and breast cancer. J Clin Med. 2016;5(2):13.

46. Stevens A, Smith H, Garner T, et al. Interactome comparison of human embryonic stem cell lines with the inner cell mass and trophectoderm. bioRxiv. 2018.

47. Murayama H, Masaki H, Sato H, Hayama T, Yamaguchi T, Nakauchi H. Successful reprogramming of epiblast stem cells by blocking nuclear localization of β-catenin. Stem Cell Reports. 2015;4(1):103-113.

48. del Valle I, Rudloff S, Carles A, et al. E-cadherin is required for the proper activation of the Lifr/Gp130 signaling pathway in mouse embryonic stem cells. Development. 2013;140(8):1684-1692.

49. Carbognin E, Betto RM, Soriano ME, Smith AG, Martello G. Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency. EMBO J. 2016;35(6):618-634.

50. Dang CV. MYC, metabolism, cell growth, and tumorigenesis. Cold Spring Harb Perspect Med. 2013;3(8):a014217.

51. Laplante M, Sabatini DM. mTOR signaling at a glance. Mol Cell Proteomics. 2010;9(12):2535-2547.

52. Stevens A, Smith H, Garner T, et al. Interactome comparison of human embryonic stem cell lines with the inner cell mass and trophectoderm. bioRxiv. 2018.
60. Lombard DB, Alt FW, Cheng HL, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol Cell Biol. 2007;27(24):8807-8814.

61. Harris TJ, Grosso JF, Yen HR, et al. Cutting edge: an in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. J Immunol. 2007;179(7):4313-4317.

62. Pawlus MR, Wang L, Hu CJ. STAT3 and HIF1alpha cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells. Oncogene. 2014;33(13):1670-1679.

63. Smith TG, Robbins PA, Ratcliffe PJ. The human side of hypoxia-inducible factor. Br J Haematol. 2008;141(3):325-334.

64. Wanet A, Arnould T, Najimi M, Renard P. Connecting mitochondria, metabolism, and stem cell fate. Stem Cells Dev. 2015;24(17):1957-1971.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Sharaireh AM, Fitzpatrick LM, Ward CM, McKay TR, Unwin RD. Epithelial cadherin regulates transition between the naïve and primed pluripotent states in mouse embryonic stem cells. Stem Cells. 2020;38:1292–1306. [https://doi.org/10.1002/stem.3249](https://doi.org/10.1002/stem.3249)