Silencing or Amplification of Endocannabinoid Signaling in Blastocysts via CB₁ Compromises Trophoblast Cell Migration

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Endocannabinoid signaling plays key roles in multiple female reproductive events. Previous studies have shown an interesting phenomenon, that mice with either silenced or elevated endocannabinoid signaling via Cnr1 encoding CB₁ show similar defects in several pregnancy events, including preimplantation embryo development. To unravel the downstream signaling of this phenomenon, microarray studies were performed using RNAs collected from WT, Cnr1⁻/⁻, and Faah⁻/⁻ mouse blastocysts on day 4 of pregnancy. The results indicate that about 100 genes show unidirectional changes under either silenced or elevated anandamide signaling via CB₁. Functional enrichment analysis of the microarray data predicted that multiple physiological functions and pathways are affected under aberrant endocannabinoid signaling. Among them, genes enriched in cell migration are suppressed in Cnr1⁻/⁻ or Faah⁻/⁻ blastocysts. Cell migration assays validated the prediction of functional enrichment analysis that cell mobility and spreading of either Cnr1⁻/⁻ or Faah⁻/⁻ trophoblast stem cells are compromised. Either silenced or elevated endocannabinoid signaling via CB₁ causes similar changes in downstream targets in preimplantation embryos and trophoblast stem cells. This study provides evidence that a tightly regulated endocannabinoid signaling is critical to normal preimplantation embryo development and migration of trophoblast stem cells.

Background: Mouse preimplantation embryo growth is retarded under elevated or silenced endocannabinoid signaling involving CB₁.
Results: Developing preimplantation embryos under these conditions have abnormal expression of migration-related genes and compromised trophoblast stem cell migration.
Conclusion: Embryo development and trophoblast migration require appropriate endocannabinoid signaling.
Significance: A tightly regulated endocannabinoid signaling threshold is critical for female reproductive success.

Endocannabinoid signaling plays key roles in multiple female reproductive events. Previous studies have shown an interesting phenomenon, that mice with either silenced or elevated endocannabinoid signaling via Cnr1 encoding CB₁ show similar defects in several pregnancy events, including preimplantation embryo development. To unravel the downstream signaling of this phenomenon, microarray studies were performed using RNAs collected from WT, Cnr1⁻/⁻, and Faah⁻/⁻ mouse blastocysts on day 4 of pregnancy. The results indicate that about 100 genes show unidirectional changes under either silenced or elevated anandamide signaling via CB₁. Functional enrichment analysis of the microarray data predicted that multiple physiological functions and pathways are affected under aberrant endocannabinoid signaling. Among them, genes enriched in cell migration are suppressed in Cnr1⁻/⁻ or Faah⁻/⁻ blastocysts. Cell migration assays validated the prediction of functional enrichment analysis that cell mobility and spreading of either Cnr1⁻/⁻ or Faah⁻/⁻ trophoblast stem cells are compromised. Either silenced or elevated endocannabinoid signaling via CB₁ causes similar changes in downstream targets in preimplantation embryos and trophoblast stem cells. This study provides evidence that a tightly regulated endocannabinoid signaling is critical to normal preimplantation embryo development and migration of trophoblast stem cells.

Cannabis is a widely used drug of abuse that profoundly alters physiological functions in the human body (1). (−)-Δ⁹-tetrahydrocannabinol (THC), the most potent psychotropic constituent of cannabis, mainly works through two G-protein-coupled receptors: cannabinoid receptor type 1 (CB₁, encoded by Cnr1) (2) and type 2 (CB₂, encoded by Cnr2) (3). Besides the natural cannabis products, endogenously produced specific cannabinoid-like compounds (endocannabinoids) are ligands for these receptors. Anandamide (AEA) and 2-arachidonoylglycerol are major endocannabinoids that are known to bind and activate these receptors, influencing multiple physiological processes (4–6). AEA, which is synthesized by multiple pathways (7–10), is primarily degraded by fatty acid amide hydrolase (FAAH) to ethanolamine and arachidonic acid (11, 12). Although N-acylethanolamine-hydrlyzing acid amidease can hydrolyze AEA (13, 14), FAAH is critical for regulating both the magnitude and duration of AEA signaling (15). Of note, in addition to AEA, FAAH also degrades other fatty acid amides, including acylethanolamides, fatty acid primary amides (oleamide) (16), and N-acyl amino acids.

AEA and CB₁ are abundant in the central nervous system but are also detected in peripheral organs, including the adrenal gland, heart, uterus, testis, and liver (17–19), whereas CB₂ is primarily present in the spleen and immune cells (19–21). In the female reproductive system, endocannabinoids, CB₁, and FAAH are present in the ovary, embryo, oviduct, uterus, and placenta. Our previous studies have shown that endocannabinoid signaling plays key roles in female reproduction (23). A tightly regulated endocannabinoid signaling is critical for the success of multiple pregnancy events, including preimplantation embryo development (24), oviductal embryo transport...
(25), implantation (26, 27), and placentation (28). Cnr1 mutant mice have been used to study the loss of function, whereas Faah mutant mice have been utilized to study amplified signaling, both via CB1 (29). However, it is to be noted that palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) levels are also elevated in Faah^−/− mice (29). Among the elevated acylethanolamides in Faah^−/− mice, only AEA has been shown to play a key role in female reproduction (30). In many instances, wild-type (WT) female mice treated with THC show similar phenotypes as seen in Faah^−/− mice, and SR141716 (a selective CB1 antagonist) attenuates the effects of THC (24). The results suggest that elevated AEA signaling via CB1 causes reproductive defects in Faah^−/− females, although the role of other acylethanolamides in female reproduction cannot completely be ruled out. Moreover, previous studies using Cnr1^−/− and Faah^−/− females have shown that both silenced and elevated AEA signaling result in similar adverse phenotypes for different pregnancy events (30). However, the mechanisms by which endocannabinoid signaling makes mouse preimplantation embryos a suitable model to study further the potential cause behind these similar phenotypes (24, 25).

To study further the mechanism by which endocannabinoid signaling executes various processes, mouse preimplantation embryos were selected for the present study because preimplantation embryos are major targets for endocannabinoid signaling, and embryo development is retarded in both Cnr1^−/− and Faah^−/− females. Therefore, similar phenotypic characteristics of embryo development with silenced or elevated AEA signaling make mouse preimplantation embryos a suitable model to study further the potential cause behind these similar phenotypes (24, 25).

To accomplish this goal, we analyzed global gene expression profiles using RNAs isolated from preimplantation blastocysts retrieved from WT, Cnr1^−/−, and Faah^−/− females. Surprisingly, we found that the expression of several dozen genes is significantly changed in one direction in both Cnr1^−/− and Faah^−/− blastocysts compared with WT blastocysts. No genes showed significantly increased expression in Cnr1^−/− blastocysts with decreased expression in Faah^−/− blastocysts compared with those in WT blastocysts or in the opposite direction. In addition, alteration of a cluster of genes known to regulate cell migration and mobility were recognized in Cnr1^−/− and Faah^−/− blastocysts, and cell migration assays using Cnr1^−/− and Faah^−/− trophoblast stem (TS) cells confirmed that cell mobility is adversely affected in these mutant cells.

**MATERIALS AND METHODS**

*Mice—*Cnr1^−/−* mice (31) were initially provided by Tom Bonner (NIMH, National Institutes of Health), whereas Faah^−/− mice (29) were obtained from Ben Cravatt (Scripps Research Institute). WT, Cnr1^−/−, and Faah^−/− mice on a C57BL/6J129 mixed background were used in this study. All mice were housed in the barrier facilities of the Animal Facility of Cincinnati Children’s Hospital Medical Center with a dark/light cycle maintained at 12 h/12 h. All protocols for animal studies were approved by the Cincinnati Children’s Research Foundation Institutional Animal Care and Use Committee consistent with National Institutes of Health guidelines.

*Blastocyst Collection for Microarray, Quantitative PCR, and Immunofluorescence Staining—*Female mice were mated with fertile males and checked for vaginal plug at 0900 h to confirm successful mating (day 1 of pregnancy = vaginal plug). To collect embryos, mice were scarified at 1500 h on day 4 of pregnancy. The blastocysts were snap-frozen in minimum volumes of M2 medium in a 1.5-mL tube and stored at −80 °C until RNA isolation.

*Microarray and Data Analysis—*RNA labeling and hybridization for microarray analysis were performed in three independent sets of blastocysts (10 blastocysts/group) with two replications. The detailed procedure has been published (32). In brief, blastocyst RNAs were labeled with Cy3-CTP. Fluorescently labeled microarray targets were prepared using a low RNA input fluorescent linear amplification kit (Agilent) with two rounds of amplifications. Cy5-CTP-labeled reference targets were produced from a mixture of Stratagene universal mouse reference RNA and MC1 cell RNA. Targets were purified using an RNeasy minikit (Qiagen) and then quantified on a NanoDrop scanning spectrophotometer (NanoDrop Technologies). Target cRNA was hybridized to the NIA mouse 44K microarray version 3.0 (whole genome 60-mer oligonucleotide arrays, Agilent Technology, design ID 015087 (33)) according to the manufacturer’s protocol (two-color microarray-based gene expression analysis protocol, product G4140–9050, version 5.0.1). Slides were scanned with an Agilent DNA microarray scanner (model G2505–64120) at 100 and 10% photomultiplier tube in both channels, with a scan resolution of 5 μm. All hybridizations compared one Cy3-CTP-labeled experimental target with the single Cy5-CTP-labeled reference target, which was used for normalization. The microarray data have been submitted to the GEO database (accession number GSE36399).

*Immunofluorescence—*Embryos or TS cells for immunofluorescence staining were fixed in freshly prepared 5% formaldehyde or 2% paraformaldehyde in PBS, followed by the protocol described previously (34). Immunofluorescence of SOX17 was performed using a Guinea pig anti-SOX17 antibody (1:1000; a kind gift from Dr. Whitsett) followed by a secondary antibody, Cy3-conjugated donkey anti-Guinea pig (706-165-148, Jackson ImmunoResearch). Actin staining was performed using rhodamine phalloidin (R415, 1:500; Invitrogen), and nuclear staining was by Hoechst 33342 (H1392, 2 μg/ml; Molecular Probes). The stained embryos or cells were visualized and imaged under a confocal microscope (Nikon Eclipse TE2000-E) and image system.

*Trophoblast Stem Cell Culture—*WT, Cnr1^−/−, and Faah^−/− TS cell lines were established and maintained as described previously (35).

*Migration Assays—*The migration assay is based on previously published reports with some modifications (28, 36). In brief, migration of TS cells was analyzed using 24-well BD BioCoat Matrigel invasion chambers. Equal numbers of WT, Cnr1^−/−, and Faah^−/− TS cells were seeded in four individual chambers each time; these experiments were repeated on two different days. The migrated TS cells were then stained by crystal violet, and the bottom membrane of the top chamber with the migrated cells was peeled off and mounted. Images were
captured under a Nikon Eclipse TS100 microscope. Cell numbers were quantified under the microscope.

**Scratch/Wound Healing Assays**—WT and mutant TS cells were seeded onto poly-1-lysine-coated coverslips. When reaching 100% confluence, TS cells were treated with mitomycin C (10 μg/ml; 2 h). The scratches were created by 20-μl pipette tips, and floating cells were removed by gentle washing. Images were taken at 0, 16, and 24 h after scratch. Images at 24 h after scratch were used to quantify the rate of cell migration.

**Attachment and Spreading Assays**—TS cells were trypsinized and resuspended in culture medium and then seeded onto poly-1-lysine-coated coverslips in culture dishes. Each time, aliquots of TS cells of different genotypes were plated in three individual culture dishes and individually monitored. Phase-contrast images were captured 4 and 24 h later. Three fields were selected randomly on each slide. The cells attached or unattached were counted by the presence or absence of the white light ring around the cells under a phase-contrast microscope. The experiments were repeated three times.

**Western Blotting**—Protein extraction and Western blotting were performed as described (35). Antibodies to PTK2B (PYK2) (sc-1514, Santa Cruz Biotechnology, Inc.), phospho-PTK2B (Tyr-402) (ab4800, Abcam), ERBB2 (ab27086, Abcam), PECAM1 (sc-1506, Santa Cruz Biotechnology, Inc.), and β-actin (sc-1615, Santa Cruz Biotechnology, Inc.) were used. β-Actin served as a loading control.

**Quantitative PCR**—About 50 blastocysts were collected and pooled together for RNA extraction, and cDNA was generated as described previously (37). Total RNA from TS cells was extracted by TRIzol reagents (Invitrogen). Quantitative PCR (qPCR) using the cDNA from embryos or TS cells was performed as described (38). The housekeeping gene Gapdh served as an internal standard. The primer sequences for each gene were as follows: Pitk2b, TCCGGAATGACTACATGCAA and CTGGCTTGGTGGATGATTTT; Erbb2, ACATGCT-TCGCCACCTCTAC and TGGTTTACCTCGGTGTGAGC; Pecam1, ATGGCCGAAGACAGACACTA and ATGGATGC-TGTTGATGTTGA; Atp5h, CTGGCTTGGTGGATGATTTT and GCTGGGATTCGCAGCAAAAA; Slc22a17, CTGCTTA-TCGGGCTTCAC and GCCGAGACAGACACAAGTA; Coro2a, CCACGCAACACCTTCTGTT and GGGTAGT-GAGGGCTCAACT; Gapdh, TGGTTTACCTACCCCAATGTTG and AGGAGAACACCTGGTCTC.

### RESULTS

**Developmental Stages of Embryos from Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> Females Selected for Analysis**—Our previous studies showed that deficiency of either CB<sub>1</sub> or FAAH results in retarded embryo development and retention of embryos within oviducts (24, 25). Cnr1<sup>−/−</sup>, Faah<sup>−/−</sup>, and WT embryos were generated by mating mutant and WT females with males of the same genotypes, and embryos were collected from oviducts and uterus of pregnant females at 1500 h on day 4 of pregnancy. As expected, most WT embryos were at the blastocyst stage (92.3%) (Table 1) and retrieved from uteri. However, a higher percentage of Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> embryos were at the morula or earlier stages (43.4% and 18%, respectively), and many of them were still in the oviduct. Because gene expression profiles greatly vary depending on the embryonic development stages, only blastocysts, not morulae, were included in gene expression analysis to better understand the effects arising solely from disrupted endocannabinoid signaling in blastocysts.

**Global Gene Expression Is Altered in Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> Blastocysts**—To identify genes and pathways affected by aberrant endocannabinoid signaling, microarray analysis was performed using RNAs from WT, Cnr1<sup>−/−</sup>, and Faah<sup>−/−</sup> blastocysts; 25,030 genes were tested using 42,066 probes. Among those genes, 241 genes (supplemental Table S1) exhibited an increased expression of 2-fold or above in those genes, 241 genes (supplemental Table S5) showed down-regulation of more than 2-fold (Fig. 1a). In Faah<sup>−/−</sup> blastocysts, expression of 145 genes (supplemental Table S3) increased at least 2-fold, whereas 168 genes were down-regulated (supplemental Table S4) more than 2-fold compared with WT blastocysts (Fig. 1b).

Further analysis revealed that whereas the expression of 53 genes increased, 51 genes were repressed in both Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> blastocysts compared with those in WT blastocysts (Fig. 1c and supplemental Table S5). Notably, when compared with WT blastocysts, no gene that significantly increased in Cnr1<sup>−/−</sup> blastocysts was down-regulated in Faah<sup>−/−</sup> blastocysts, and vice versa (Fig. 1c). Because the endocannabinoid signaling is silenced in Cnr1<sup>−/−</sup> mice and elevated in Faah<sup>−/−</sup> mice, one might anticipate that downstream signaling pathways in these two groups would show changes in the opposite direction. Instead, the results show that both silenced and amplified AEA signaling via CB<sub>1</sub> cause changes in gene expression in a similar direction. These results may explain the reason for our previous observations that similar phenotypes are noted in Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> mice (24, 25).

**Quantitative RT-PCR and Immunofluorescence Staining**

**Validate Microarray Data**—In order to validate microarray results, several genes (Pitk2b, Pecam1, Erbb2, Atp5h, Slc22a17, and Coro2a) were analyzed by real-time PCR (qPCR) using RNAs from Cnr1<sup>−/−</sup>, Faah<sup>−/−</sup>, and WT blastocysts. Down-regulated expression of Pitk2b, Pecam1, and Erbb2 in Cnr1<sup>−/−</sup> and Faah<sup>−/−</sup> blastocysts as detected by microarray results
To analyze further the biological significance of differentially expressed genes in blastocysts detected by microarray analysis are translated. (Fig. 2a) was validated by qPCR as shown in Fig. 2b. Similarly, up-regulated expression of Atp5h, Slc22a17, and Coro2a in these mutant blastocysts, as observed in microarray analysis (Fig. 2c), was also validated by qPCR (Fig. 2d). These results suggest that microarray analysis was able to identify differentially expressed genes in blastocysts of different genotypes.

Embryos at the blastocyst stage primarily consist of two major cell types: the inner cell mass (ICM) and the trophoderm (TE). To investigate further the cell type-specific localization of proteins encoded by a specific gene, immunofluorescence staining was performed. Localization of PTK2B, ERBB2, and PECAM1 was noted in both the ICM and TE cells, whereas SOX17 was localized in ICM cells (Fig. 2e). Whereas PECAM1 was localized on the cell membrane, SOX17 localization was restricted to nuclei. In addition, the expression levels of SOX17 in WT blastocyst were higher than those in Cnr1−/− and Faah−/− blastocysts. These data show that specific genes detected in blastocysts by microarray analysis are translated.

Aberrant Blastocyst Endocannabinoid Signaling Modulates Expression of Genes Involved in Multiple Signaling Pathways—To analyze further the biological significance of differentially expressed genes in Cnr1−/− and Faah−/− blastocysts, genes were categorized into seven groups: 1) genes up-regulated in Cnr1−/− blastocysts; 2) genes down-regulated in Cnr1−/− blastocysts; 3) genes up-regulated in Faah−/− blastocysts; 4) genes down-regulated in Faah−/− blastocysts; 5) genes up-regulated in both Cnr1−/− and Faah−/− blastocysts; 6) genes down-regulated in both Cnr1−/− and Faah−/− blastocysts; and 7) combinations of down-regulated and up-regulated genes in both Cnr1−/− and Faah−/− blastocysts. The ToppFun functional enrichment analysis (39) was performed to categorize genes by enriched biological processes in gene ontology, mouse phenotypes, pathways, gene coexpression, and protein interactions. The results are listed in Table 2. In group 7, six genes are enriched for the mouse phenotype “abnormality of immune system physiology,” and three genes show enrichment for the
ifenoxin-γ signaling pathway. Of the genes up-regulated in Faah−/− blastocysts, seven genes are enriched for translational termination, and eight genes show enrichment for cellular macromolecular complex disassembly. The genes down-regulated in Cnr1−/− blastocysts showed enrichment for cell migration (17 genes), gland development (15 genes), and EGFR interactions (15 genes). Overall, the result suggests that multiple biological processes, including cell migration, immune responses, and growth factor signaling, are affected in the absence of blastocyst Cnr1 and Faah.

**Cell Migration-related Genes Are Suppressed in Cnr1−/− and Faah−/− Blastocysts**—Among the functional groups described above, differentially expressed genes in the cell migration group are most significant (Table 2). The fact that cell migration-related genes were suppressed in Cnr1−/− blastocysts is consistent with our previous findings that the Cnr1−/− trophoblast cells have reduced invasive ability during placentation in vivo (28). This correlation prompted us to perform more functional studies on cell migration. Because either silenced or augmented AEA signaling via CB1 shows similar phenotypes, we examined whether the expression levels of the same group of genes are lower in Faah−/− blastocysts. Indeed, we found that most of the genes in this category were down-regulated in Faah−/− blastocysts (Table 3). However, it is possible that the TopFun analysis failed to identify this cell migration group using down-regulated genes in Faah−/− blastocysts, because genes with 2-fold or more changes were selected. Collectively, the data suggest that cell migration in both Cnr1−/− and Faah−/− blastocysts is defective.

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### TABLE 2

Functional categorization of differentially expressed genes in Cnr1−/− and Faah−/− blastocysts using TopFun functional enrichment analysis

| GO term pathways/phenotype | Genes | Cnr1−/− | Faah−/− | Ratio of gene expression levels | Cnr1−/−/Faah−/− |
|---------------------------|-------|---------|---------|--------------------------------|-----------------|
| Abnormality of immune system physiology | Cntr, Mapk10, Syp110, Scd2, Traf3, Tp53, Psa2p7 | 3.92 | 2.91 | 7.67 |
| Interferon-gamma signaling pathway | Mapk10, Socs3, Socs4 | 3.92 | 2.91 | 7.67 |
| Chromatid body | Ddx4, Ddx5, Mecl | 3.92 | 2.91 | 7.67 |
| Methylation-like degradation | Gja1, Hgfl | 3.92 | 2.91 | 7.67 |

### TABLE 3

- Fold decreases in expression levels of migration-related genes in Cnr1−/− and Faah−/− blastocysts compared with WT blastocysts in microarray data

| Cell migration-related genes | Cnr1−/− | Faah−/− | Ratio of gene expression levels |
|-----------------------------|---------|---------|--------------------------------|
| Igif1 | 6.21 | 7.67 |
| Ntn1 | 7.19 | 5.88 |
| Igif1 | 17.51 | 2.74 |
| Lama1 | 9.42 | 2.70 |
| Ptk2b | 4.14 | 2.59 |
| Plau | 5.48 | 2.44 |
| Pecam1 | 4.51 | 2.23 |
| Adora1 | 28.68 | 2.08 |
| Nod2 | 35.29 | 1.98 |
| Fgfl | 12.54 | 1.94 |
| Apoe | 3.92 | 1.87 |
| Vegfc | 21.60 | 1.70 |
| Tgfbl | 4.49 | 1.69 |
| Erbb2 | 2.37 | 1.67 |
| Pdgfrb | 5.21 | 1.36 |
| Plgfra | 2.93 | 1.04 |
| Pdhl1 | 15.93 | 0.91 |

- After implantation of the blastocyst, the ICM develops into the trophectoderm and inner cell mass. The trophectoderm cells differentiate into the extraembryonic membranes, whereas the inner cell mass forms the embryonic disk, which will give rise to the embryo proper, whereas the trophoblast cells penetrating through the epithelium migrate to the stroma and ultimately invade into the maternal decidua, forming the placenta (40). During implantation and placentation, trophoblast invasion and migration are tightly regulated (41). To study whether cell mobility is affected by the aberrant endocannabinoid signaling, cells were transmigrated from the upper to the lower chamber of a Boyden chamber transmigration assay. Cells seeded onto the upper chamber with low levels of fetal bovine serum (FBS) transmigrated to the lower chamber 2-fold or more changes were selected. Collectively, the data suggest that cell migration in both Cnr1−/− and Faah−/− blastocysts is defective.
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FIGURE 3. Cnr1⁻/⁻ and Faah⁻/⁻ TS cells show compromised migration. a, in vitro migration assays show that numbers of Cnr1⁻/⁻ and Faah⁻/⁻ TS cells successfully migrating to the lower chamber of Boyden chambers are much lower than those of WT TS cells after 44 h in culture. b, quantification of cell migration as shown in a (unpaired t test, n = 8; *, p < 0.05). c, representative phase-contrast images of in vitro wound healing assays. Wound edges are demarcated by red dotted lines. WT TS cells closed the wound gap faster than Cnr1⁻/⁻ and Faah⁻/⁻ TS cells. Scale bar, 500 μm. d, quantification of cell migration in wound healing assays in c (unpaired t test; *, p < 0.05). Error bars, S.E.

FIGURE 4. Cnr1⁻/⁻ and Faah⁻/⁻ TS cells show compromised attachment and spreading. a, representative phase-contrast images of Cnr1⁻/⁻, Faah⁻/⁻, and WT TS cells 4 and 12 h after seeding. Significantly lower numbers of Cnr1⁻/⁻ and Faah⁻/⁻ TS cells are much lower compared with WT TS cells (unpaired t test, n = 9; *, p < 0.05). b, Cnr1⁻/⁻ and Faah⁻/⁻ TS cells remain round even after seeding for 4 h, whereas WT TS cells rapidly attach and spread out, as evident from their polygonal shape. The TS cell boundary is outlined by F-actin staining. Error bars, S.E.

the scratch. WT TS cell migrated rapidly to fill up the gap created by wounding and closed the gap within 24 h. However, Cnr1⁻/⁻ and Faah⁻/⁻ TS cells migrated much slower than WT TS cells. Surprisingly, very limited migration of Faah⁻/⁻ TS cells was observed. The results from these two migration assays suggest that aberrant endocannabinoid signaling affects TS migration, which is consistent with our gene expression data.

**TS Cell Attachment and Spreading Are Severely Compromised by Aberrant Endocannabinoid Signaling**—Normal cell migration requires dynamic assembly and disassembly of focal adhesions (43), which are associated with cell attachment and spreading (44). Lower levels of the expression of genes involved in migration in Cnr1⁻/⁻ and Faah⁻/⁻ blastocysts suggest that Cnr1⁻/⁻ and Faah⁻/⁻ TS cells have impaired ability to attach and spread. For example, the levels of PTK2b, also known as focal adhesion kinase 2 (FAK2), are involved in focal adhesion formation and associated with cell spreading (44). Our experience with generating the Cnr1⁻/⁻ TS cell line also indicated that cell attachment is severely compromised. Cnr1⁻/⁻ TS cells take a much longer time to attach onto the culture dish after each passage. Four hours after seeding, WT TS cells started to spread out and became flat, as indicated by the reduced reflection of light under a phase-contrast microscope (Fig. 4a), and via actin fibers that outlined the polygonal-shaped WT cells that had undergone spreading (Fig. 4c). However, Cnr1⁻/⁻ and Faah⁻/⁻ TS cells were still round, as indicated by white light rings around the cells (Fig. 4a), and actin fibers formed a red ring around the cells (Fig. 4c). Even after 24 h, not all Cnr1⁻/⁻ and Faah⁻/⁻ TS cells were completely attached (Fig. 4, a and b).
Some Cnr1−/− and Faah−/− cells failed to attach and ultimately died. These results show that either silenced or augmented AEA signaling via CB1 compromises cell attachment.

Cell Migration- and Locomotion-related Genes Are Suppressed in Cnr1−/− and Faah−/− TS Cells—The functional enrichment analysis of microarray data predicted compromised cell migration based on the expression of a cluster of genes related to migration. Using TS cells as a model system, cell migration and wound healing assays confirmed the result of the functional enrichment analysis. We then asked whether the gene expression pattern in the microarray data is reflected in TS cells used for the functional assays. Western blotting experiments were performed using protein lysates from WT, Cnr1−/−, and Faah−/− TS cells. The results show that protein levels of migration-related genes, such as Ptk2b, Pecam1, and Pld1, were lower in Cnr1−/− and Faah−/− TS cells compared with WT TS cells (Fig. 5a). The normalized quantitative data are shown in Fig. 5b. The expression of ERBB2 from the group of genes related to gland development was also examined in TS cells. These results corroborate the blastocyst microarray data (Table 3 and Fig. 2a). Collectively, the findings show that compromised mobility of Cnr1−/− and Faah−/− TS cells is associated with depressed expression of migration-related genes.

DISCUSSION

Endocannabinoid signaling plays key roles in regulating multiple biological functions, including synaptic plasticity (45), pain modulation (46, 47), energy homeostasis (48), and reproduction (30, 49). Recently, several studies have also suggested that cannabinoid signaling plays a role in cell migration using cell lines, although the results remain controversial (50, 51). Other fatty acid amides that affect cell migration include PEA (52) and oleamide (53, 54). In female reproduction, both Cnr1−/− and Faah−/− mice have been used to study the role of endocannabinoid signaling in different pregnancy events (24, 25, 55). In many cases, similar phenotypes were observed in females with either silenced or elevated AEA signaling via CB1, suggesting that a tightly regulated signaling is required for normal female reproductive processes and that changes in downstream signaling targets under silenced or elevated AEA signaling are similar.

In this study, we sought to explore why endocannabinoid signaling functions through such a narrow window of signaling threshold to influence female reproduction. Using preimplantation blastocyst as a model system, global gene microarray studies revealed that 53 genes were up-regulated and 51 genes were down-regulated in both Cnr1−/− and Faah−/− blastocysts. Surprisingly, no gene was found to show changes in opposite directions in Cnr1−/− and Faah−/− blastocysts compared with WT blastocysts.

There is now evidence that tissue levels of PEA and OEA in addition to AEA are also elevated in Faah−/− mice (29), suggesting overall increases in levels of acylethanolamides in Faah−/− mice. PEA, mainly targeting peroxisome proliferator-activated receptors, exerts a great variety of biological functions related to chronic pain and inflammation (56), whereas OEA can modulate feeding behavior, body weight, and lipid metabolism (57). Although OEA and PEA have been shown to have roles in male reproduction (58, 59), their roles in female reproduction are still elusive.

Among elevated acylethanolamides in Faah−/− mice, only AEA has been shown to play a key role in female reproduction (30). In many respects, WT females treated with THC show similar phenotypes as seen in Faah−/− mice, and SR141716 (a selective CB1 antagonist) blocks the effects of THC (24). With respect to the regulation of trophoblasts, previous studies have shown that AEA is capable of influencing trophoblast outgrowth and fibronectin-binding activity, which is increased at the adhesive stage of trophoblasts prior to invasion (60). Higher AEA levels decrease the fibronectin-binding activity of blastocysts, suggesting that increased AEA levels in Faah−/− females influence trophoblast invasion. Another study showed that lower levels of AEA (7 nM) but not higher levels activate MAPK signaling in trophoblasts (61), suggesting that AEA plays an important role in regulating trophoblast behavior. Taken together, the results suggest that elevated AEA signaling via CB1 causes reproductive defects in Faah−/− females, although roles of other acylethanolamides in female reproduction cannot be ruled out.

The microarray data show that there is a trend in changes of gene expression toward one direction under either silenced or elevated AEA signaling. This unique feature of endocannabinoid signaling in preimplantation embryo development and perhaps in other female reproductive events makes this signaling a high fidelity sensor in that a deviation from the physiological threshold of endocannabinoid signaling may lead to female subfertility. Whether this feature of endocannabinoid signaling is also applicable to other organ systems is not known. This observation is surprising and unique because higher and silenced AEA signaling normally shows contrasting effects on other physiological events like retrograde synaptic depression in the cerebellum (62), motivation for food (63, 64), and analgesic effects (31). Although the nature and role of the secondary messengers of CB1 mediating blastocyst growth and trophoectoderm migration for implantation are not clearly understood, MAPK could be one such secondary messenger that is influenced by CB1 signaling during implantation (61). The mechanism by which either silenced or elevated AEA signaling in preimplantation embryos shows similar effects on downstream signaling warrants further investigation.
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Functional enrichment analysis of the microarray data using the ToppFun application of ToppGene Suite (39) further predicted that gene expression changes under aberrant endocannabinoid signaling would affect multiple biological processes, including cell migration, gland development, EGFR interactions, translational termination, and interferon-γ signaling. However, the functional enrichment analysis did not indicate any major biological function using genes unidirectionally changed in both Cnr1−/− and Faah−/− blastocysts. Among the functionally categorized groups of genes, the cell-migration-related group was derived from the analysis of down-regulated genes in Cnr1−/− blastocysts but not from down-regulated genes in Faah−/−. Because only genes whose expression was changed at least 2-fold in the mutant blastocysts compared with WT blastocysts were used for functional enrichment analysis, it is possible that some functionally significant genes were excluded from the functional analysis. In fact, when we further analyzed using a lower cut-off level of -fold changes of migration-related genes in the pool of down-regulated genes in Faah−/− blastocysts, most of the genes were found to be down-regulated in Faah−/− blastocysts compared with WT blastocysts (Table 3). In addition, the cell migration assays confirmed that cell mobility was compromised in both Cnr1−/− and Faah−/− TS cells.

Although Cnr1−/− and Faah−/− preimplantation embryos show retarded embryo development, the gene set enrichment analysis of differentially expressed genes did not show any enrichment of biological processes or pathways critical to embryo development. Two possible reasons are 1) that morula collected from Cnr1−/− and Faah−/− females were excluded, and thereby changes in expression of genes related to embryo development were modest in Cnr1−/− and Faah−/− blastocysts; and 2) that the hit number, which is the overlapping pool of significantly altered embryo developmental genes compared among the seven groups listed above, was low, thus precluding the group from being significant. However, the aberrant expression of a few genes that are critical to embryo development may be enough to cause retarded embryo development. In fact, several genes critical to embryo development were repressed in both Cnr1−/− and Faah−/− blastocysts when compared with WT blastocysts. For example, Eomes, which is required for mouse trophoblast development (66), was down-regulated by 65 and 22% in Cnr1−/− and Faah−/− blastocysts, respectively, compared with WT blastocysts. In addition, transcription factor Spt1, which is essential for early embryonic development (67), was down-regulated in Cnr1−/− and Faah−/− blastocysts by 76 and 42%, respectively. Notably, expression of Dpp1 (68) and Hormad1 (69), which play roles in embryo development, was also lower in Cnr1−/− and Faah−/− blastocysts.

The fact that the cell migration-related gene group showed the most significant changes in Cnr1−/− and Faah−/− is very interesting in light of our previous observations that Cnr1−/− trophoblasts exhibited impaired invasion during placentation (28). Moreover, difficulties in generating Cnr1−/− TS cell lines also suggest that these TS cells had an impaired ability to anchor onto the culture dishes. We were unsuccessful in generating a Cnr1−/− TS cell line for about 2 years until recently. Cnr1−/− TS cells were expanded in first several passages by separating big colonies of TS cells into small patches comprising only 2–5 TS cells using a sterile needle. A large population of Cnr1−/− TS cells was lost due to an inability of these cells to attach to culture dishes during the first several passages, and expansion and maintenance of the Cnr1−/− TS cells became very difficult. In conclusion, the results of this study showing down-regulation of genes linked to cell migration corroborate our previous in vivo and vitro studies that showed shallow invasion of placentas in Cnr1−/− and Faah−/− females (28). Collectively, these results not only suggest that the endocannabinoid signaling is critical for trophoblast function; they also suggest that the programming of trophoblast cells by endocannabinoids very early in development has adverse effects on placentation later in pregnancy, thus compromising pregnancy outcome.

Studies in TS cells have great clinical importance regarding the onset of preeclampsia in pathologic pregnancy (65), because trophoectoderm forms the majority of the placenta, and abnormal placentation is a common feature in preeclampsia. Although the cause of preeclampsia still remains elusive, trophoblast invasion plays a key role in placentation (22). Under aberrant endocannabinoid signaling, defects in trophoblast cells seeded at the blastocyst stage may be reflected later during placentation. The present investigation shows that the mobility and migration of Cnr1−/− and Faah−/− TS cells are compromised, suggesting that these mutant mice are potential models to study preeclampsia. This is, in fact, consistent with our previous findings showing shallow invasion of Cnr1−/− trophoblast cells into the maternal decidua (28).

The findings of this investigation raise concerns that exposure to cannabis products may adversely affect early embryo development that is then perpetuated later in pregnancy. Given that endocannabinoid signaling plays a key role in the central nervous system, it would be interesting to examine whether cell migration-related genes as identified in blastocysts also participate in neuronal migration during brain development.

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