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Analysis for Carom complex, signaling and function by database mining

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1. ABSTRACT

Carom is a novel protein that regulates membrane curvature and transmits pathophysiological signaling. The tissue expression of Carom is unclear and its functional role and signaling are unknown. We employed a group of combined database mining strategies and established a working model of Carom signaling. We identified 26 Carom partners and established their expression profiles in human and mouse tissues. We classified three tiers of tissues for Carom/partner expression and found lymph node was the tier 1 tissue expressing Carom and most of its partners. Using GEO database, we discovered that four conditions (hypoxia, endometriosis, PPARγ deletion and iPSC reprogramming) altered Carom/partner expression in endothelial cells. We identified 26 Carom partner signalings by Ingenuity pathway analysis. Ten of the 26 pathways and three genes (ITSN1, UBC and HSPA5) were reported to be regulated in the above four conditions. Paired induction of Carom/ITSN1 elevation was associated with pathological angiogenesis. Whereas, paired reduction of Carom/HSPA5 or UBC was associated with iPSC generation. These results provide an insight on identifying Carom complex model and predicting its functional implications.

2. INTRODUCTION

Cell membrane curvature modulates many important cellular processes, including endocytosis, phagocytosis, adhesion, angiogenesis and migration.

Changes of cell membrane shape are modified by cytoskeleton and membrane coordinating proteins. Recently, the FES/CIP4 homology-Bin/Amphiphysin/Rvs (F-BAR) protein family has been identified as important coordinators for membrane curvatures (1-3). F-BAR proteins bind to cell membrane via N-terminal F-BAR domain, which associates with membrane phospholipids (4-6). Most F-BAR proteins contain at least one C-terminal Src homology-3 (SH3) domain to interact with actin-associated proteins, like Wiskott-Aldrich syndrome protein (WASP) and WASP family verproline-homologous protein (WAVE) (7). This interaction leads to actin polymerization and causes various membrane curvatures.

Based on the domain structure of F-BAR proteins, we classified mammalian F-BAR family into nine subfamilies. These include 1) CDC42-interacting protein 4 (CIP4), 2) FCH only (FCHO), 3) Slit-Robo GTPase-activating protein (srGAP), 4) protein kinase C and casein kinase 2 substrates in neurons (PACSIN), 5) proline-serine-threonine phosphatase-interacting protein (PSTPIP), 6) FCH and double SH3 domain proteins (FCHSD), 7) FES/FES-related (FER), 8) nitric oxide synthase traffic inducer (NOSTRIN), and 9) growth arrest-specific 7 (GAS7) subfamilies (8). Carom is a novel member of F-BAR family protein in the FCHSD subfamily, and encoded by gene FCHSD2. There are few research reported concerning Carom protein. The tissue expression profile of Carom has not been studied. The protein complex of Carom and its signaling pathways remains unknown.

In the past ten years, bioinformatics analysis has become an important tool for functional interpretation of genomics and proteomics prediction. We have established several database mining strategies to determine functional relationship of genes in specific metabolic groups by using National Institutes of Health (NIH)/National Center of Biotechnology Information (NCBI) Gene database and Expression Sequence Tag (EST) database, Gene Expression Omnibus (GEO) database and Transcription Element Search System (TESS) database (9-18). We also established various working models to predict and explain mechanisms underlying cardiovascular and inflammatory diseases (9, 12, 14, 16).

However, bioinformatics analysis has not been used to analyze protein-protein interaction prediction. In this study we developed a novel database mining strategy to determine Carom complexes and signaling pathways by using the combination of multiple national database and software system, including NCBI Gene database and EST database, GEO database, Ingenuity pathway analysis (IPA) software and PubMed literature reviews. We identified Carom partners, presented a comprehensive analysis of tissue mRNA distribution profiles of Carom and partners in human and mouse tissues, analyzed conditions with altered Carom signaling and partner expression changes in various cells, and finally developed a working model of Carom protein complex in physiological and pathophysiological processes.

3. MATERIALS AND METHODS

3.1. Identification of Carom partners (NCBI Gene database)

As previously described, an experimental data mining strategy was used to establish tissue expression profiles of Carom and its interacting partners (16, 14, 11) (Figure 1). A total of 26 binding partners for Carom protein were identified by mining NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene/9873) (Table 1). These databases provided information for partnership determined via the following experimental approaches. (1) Two-hybrid identify the protein-protein interaction by transcription factor activation initiated by fusion of the factor's DNA binding domain and its transcriptional activation domain (19). (2) Affinity Capture-Mass spectrometry (MS) analysis determine the interaction when a bait protein is captured by either a specific antibody or an epitope tag and the interaction partner is analyzed via mass spectrometry (20). (3) Co-immunoprecipitation identify the interaction when a bait protein is immunoprecipitated by specific antibody and the interacting protein is also co-immunoprecipitated and identified by Western Blot (21). (4) Affinity Capture-RNA recognize the interaction for RNA-binding proteins when a bait protein is affinity captured by either a specific antibody or an epitope tag and associated RNA species are identified by Northern blot, RT-PCR, affinity labeling, sequencing, or microarray analysis (22).

3.2. Tissue mRNA distribution profiles of Carom and partners (EST database)

A total of 21 human and 20 mouse tissues were given tissue ID numbers and examined for mRNA expression by mining human and mouse EST databases deposited in the NIH UniGene database (23) (http://www.ncbi.nlm.nih.gov/unigene). The EST database is created via cDNA cloning from various tissue cDNA libraries followed by DNA sequencing. As previously described, we used relative mRNA expression units (REU) of Carom and binding partners, normalized by the transcripts per million (TPM) of Carom and binding partners with that of β-actin (left-side y-axis). Further, we determined the median REU (mREU) of the human and mouse tissues in order to compare the gene expression. The ratio of REU/mREU was expressed as tissue median adjusted mRNA expression units and presented in Figure 3A (right-side y-axis). A confidence interval for the gene expression was generated using mean REU and 2 times the standard deviation (SD) (mean ± 2SD) of the REU of 3 randomly selected housekeeping genes (pituitary tumor-transforming 1 interacting protein (PTTG1IP), pyruvate kinase muscle (PKM2), and heterogeneous

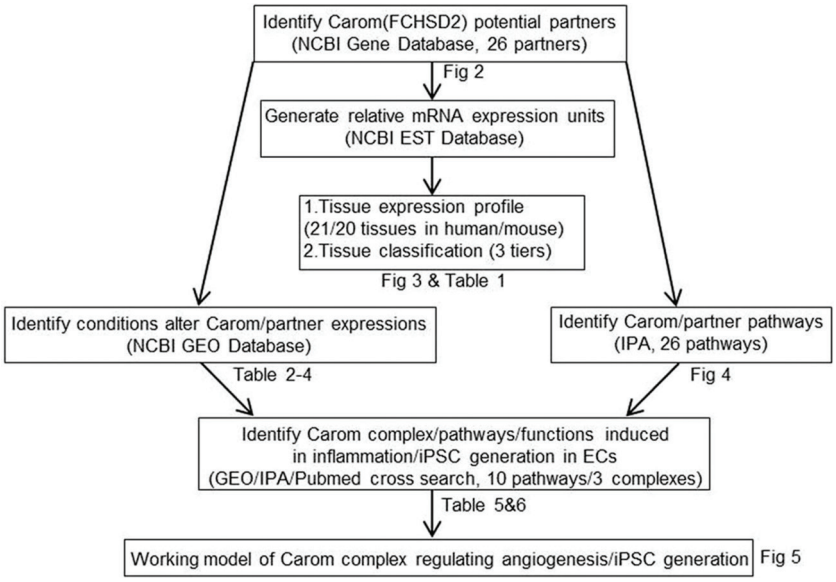

Figure 1. Flow chart of database mining strategy. 26 genes were identified as the partners of Carom (gene name: FCHSD2) from NCBI Gene Database (Fig 2). Relative mRNA expression units and tissue expression profiles were generated using NCBI EST database (Fig 3). Tissues were classified based on the expression of Carom/partners (Table 1). Inflammation and induced pluripotent stem cells (iPSC) generation were identified as conditions where Carom/partners gene expression altered in endothelial cells (ECs) by querying GEO datasets (Table 2-4). 26 signaling pathways were recognized potentially involved in Carom/partners signaling by using Ingenuity pathway analysis (IPA) software (Fig 4). 10 of these 26 pathways were reported in Pubmed and 3 Carom complexes and its function were identified by GEO/IPA/Pubmed cross search (Table 5&6). A working model Carom complex in regulating angiogenesis and iPSC generation in ECs was generated (Fig 5). Abbreviations: ECs, Endothelial cells; EST: Expressed sequence tags; GEO, Gene expression omnibus; IPA, Ingenuity pathway analysis; iPSC, induced pluripotent stem cells.

nuclear ribonucleoprotein K (HNRNPK)) in 21 human or 20 mouse tissues, normalized by β-actin in given tissues. If the expression variation of a given gene in the tissues was larger than the upper limit of the confidence interval of the housekeeping genes, the high expression levels of genes in the tissues were considered statistically significant.

3.3. Identification for conditions altering Carom expression (GEO database)

To examine the differential expression profiles of Carom in response to various experimental conditions, we searched the high-throughput functional genomics datasets in NCBI/GEO database (http://www.ncbi.nlm.nih.gov/gds). The datasets enabled us to identify the expression changes of Carom in endothelial cells (ECs), monocytes, macrophages, smooth muscle cells and epithelial cells from 19 datasets involving three experimental conditions including inflammatory, reprogramming and angiogenesis (Table 3). If the fold changes of gene expression were ≥1.5. with P <0.0.5 in treated cells, it was considered a significant increase. Gene expression fold changes ≤ 0.8. with P <0.0.5 were considered a significant decrease.

3.4. Pathway analysis of Carom and partners (Ingenuity pathway analysis)

Carom and 26 binding partners were analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) to predict the molecular networks and signaling pathways of Carom complex. Significance of association between sets of genes and related molecular and cellular functions was assessed using the Fisher's Exact Test. Significant Carom complex signal pathways were selected and presented in Figure 4 based on a default threshold of IPA (-log (p-value) >1.3.) meaning that P value <0.0.5.

3.5. Predicted function of Carom complex signaling (PubMed)

In order to identify the functional connection of Carom complex, we screened Pubmed information for reported Carom signaling regulation in the identified 26 pathways from IPA analysis. Four conditions were used for this screening based on GEO analysis data presented in Table 4 (hypoxia, endometrial, PPARγ depletion and iPSC reprogramming). Pubmed ID related with reported Carom complex signaling pathway regulation and changed Carom partner were presented.

4. RESULTS

4.1. Identification of 26 Carom partners

A total of 26 genes were identified as Carom (FCHSD2) partners from NCBI Gene database (Table 1). The partnership was recognized by various experimental systems, including Two-hybrid, affinity Capture-MS, co-immunoprecipitation and affinity Capture-RNA. These

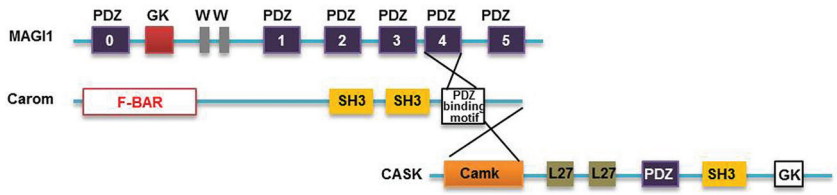

Figure 2. MAGI1 and CASK competitively binds to Carom. CASK and MAGI1 were identified as Carom binding proteins by using two-hybrid and Affinity Capture-Western in Madine Darby canine kidney (MDCK) cells. Carom binds to the PDZ domain 4 of MAGI-1 and the Camk domain of CASK.

Table 1. Identification of Carom partners (NCBI gene database)

Gene	Protein function	Approaches for interaction identification	PMID ID#
C2ORF73	N/A	Two-hybrid	18654987
C9ORF78	Associate with hepatocellular carcinoma	Two-hybrid	18654987
CASK	Regulate cell junctions	Co-immunoprecipitation	14627983
CCDC85B	Regulate cell adhesion	Two-hybrid	16189514
CCDC104	Regulate ciliary membrane	Two-hybrid	16189514
CCDC158	Associated with kidney function	Two-hybrid	16189514
CCT2	Regulate cilium formation	Two-hybrid	18654987
CHKA	Regulate phospholipid biosynthesis	Two-hybrid	18654987
DAPP1	Regulates B-cell antigen receptor signaling	Two-hybrid	21988832
ELAVL1	Regulate embryonic stem cells differentiation	Affinity Capture-RNA	19322201
GRASP	Regulate intracellular trafficking	Two-hybrid	12586822
HSP90AB1	Regulate cell cycle	Two-hybrid	18654987
HSPA5	Assemble multimeric protein complexes in endoplasmic reticulum	Two-hybrid	18654987
HTRA1	Regulate MMP1 and MMP3 production	Two-hybrid	15101818
ITSN1	Regulate endocytic vesicle formation	Two-hybrid	22558309
ITSN2	Regulate endocytic vesicle formation	Two-hybrid	22558309
MAGI1	Regulate cell junctions	Co-immunoprecipitation; Two-hybrid	14627983
MSL1	Regulate majority of histone H4 acetylation	Two-hybrid	18654987
SH3BGR	Candidate gene for Down's syndrome heart defects	Two-hybrid	18654987
SRP72	Regulate the targeting of secretory proteins to endoplasmic reticulum	Two-hybrid	18654987
UBC	Regulate protein ubiquitination	Affinity Capture-MS	24816145
UBD	Regulate protein ubiquitination	Affinity Capture-MS	22797925
VCP	Regulate vesicle trafficking	Co-immunoprecipitation Two-hybrid	18654987
VCPIP1	Interact with VCP for Golgi stacks resemble	Two-hybrid	18654987
WAS	Regulate actin polymerization	Two-hybrid	21988832
WASL	Regulate actin polymerization	Two-hybrid	21988832
26 genes were identified as the potential partners of Carom via Two-hybrid, Affinity Capture-MS, Co-immunoprecipitation and Affinity Capture-RNA from NCBI Gene Database (http://www.ncbi.nlm.nih.gov/gene/9873)			

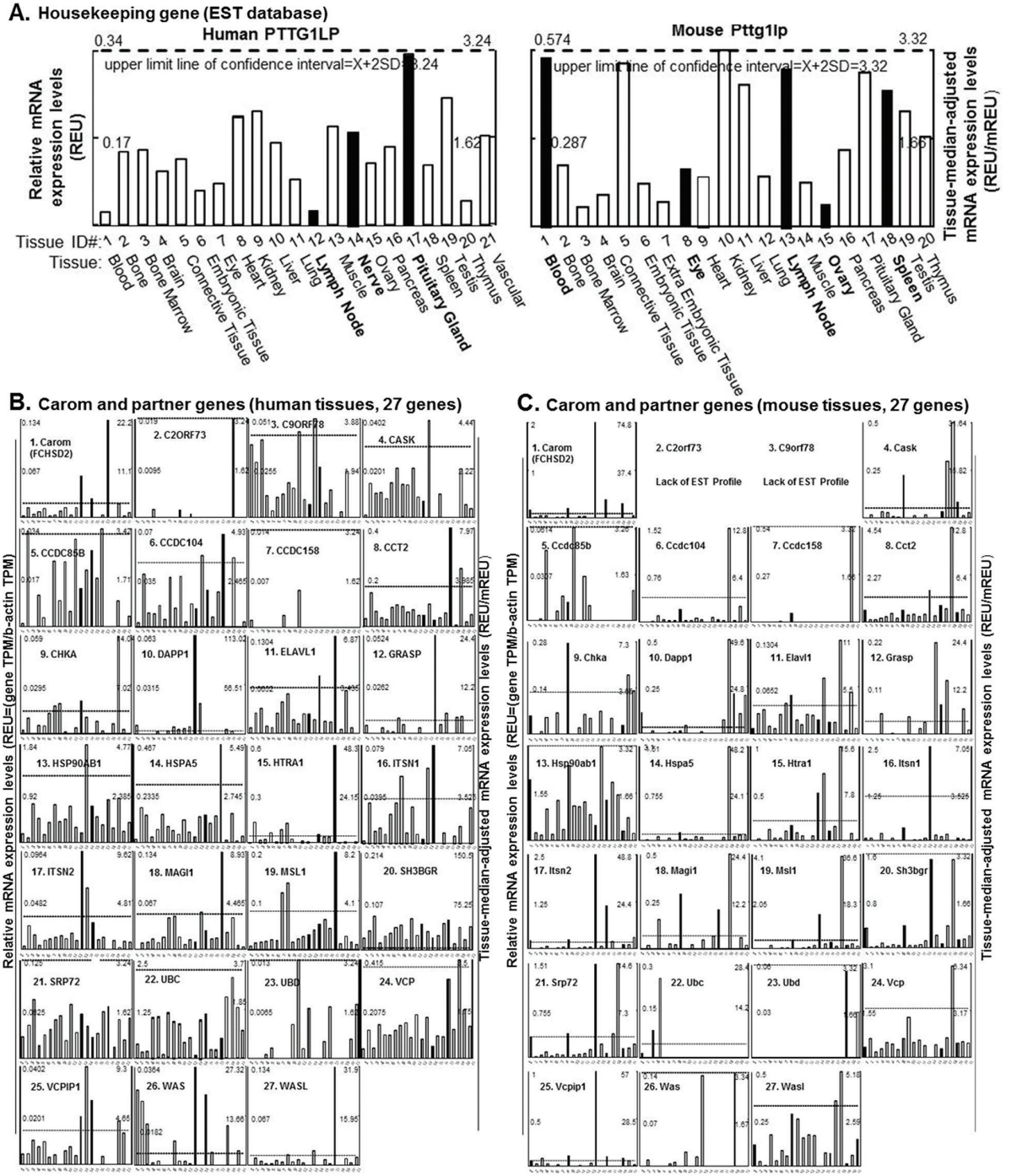

Figure 3. Tissue mRNA distribution profiles of Carom and partners (EST database). 21 human and 20 mouse tissues were given tissue ID numbers and examined for mRNA expression by mining human and mouse EST database on the NCBI-UniGene site (http://www.ncbi.nlm.nih.gov/unigene). REU of the gene is obtained by normalizing gene TPM with that of β-actin. Tissue median-adjusted mRNA expression levels (REU/mREU) were calculated for all genes. Confidence intervals of expression of three housekeeping gene mRNAs were established. Dashed lines, upper limits of the confidence intervals of the housekeeping gene. Left and right y axes, REU and REU/mREU, respectively. A. Representative tissue mRNA distribution profile of housekeeping gene PTTG1LP in human and mouse. See Methods and Materials for details. B&C. mRNA distribution profiles of Carom and 26 partner genes in 21 human tissues. D&E. mRNA distribution profiles of Carom and 26 partner genes in 20 mouse tissues. The statistical significance was defined when gene expression was larger than the upper limit of the confidence interval. Confidential interval of the gene expression was generated using mean REU and 2 times the standard deviation (SD) (mean ± 2SD) of the REU of 3 randomly selected housekeeping genes. Solid bars highlight gene expressions in tissues where Carom expression were statistically significant. Carom is highly expressed in lymph node, pituitary gland and nerve of human tissues and highly expressed in blood, eye, lymph node, spleen and ovary of mouse tissues. Abbreviations: PTTG1LP, pituitary tumor-transforming 1 interacting protein; REU, mRNA expression units; mREU, median REU; TPM, transcripts per million.

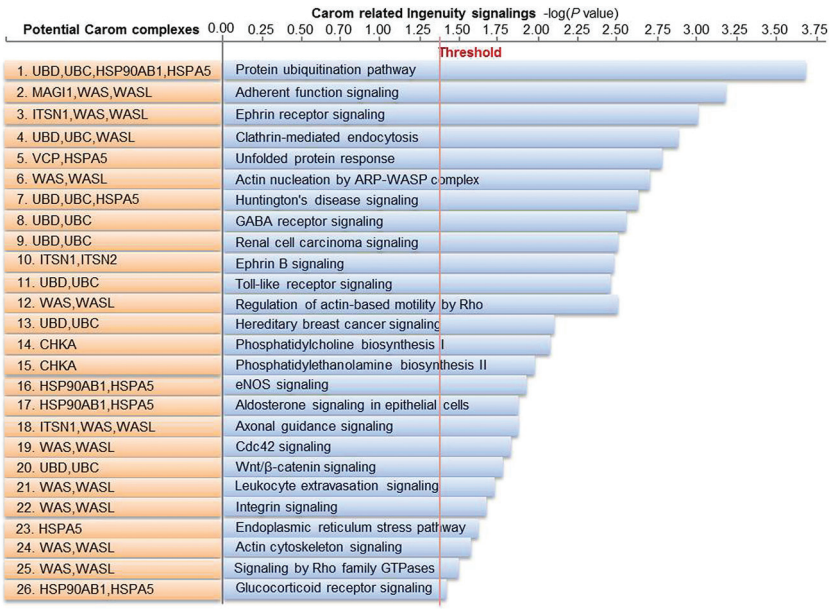

Figure 4. Identified Carom complex signal pathways (Ingenuity pathway analysis). 26 signal pathways were identified based on reported functional study of potential Carom partners using Ingenuity pathway analysis (IPA). These pathways may lead to Carom functional destinies. Threshold: -log (*P* value) >1.3. meaning that *P* value <0.0.5.

Table 2. Tissue classification of carom/partner expression

Tissue	Carom/partner expression
A. Human tissues	
Tier 1 "Ready to go", express carom and partners	
Lymph node	Carom, CHKA, DAPP1, ITSN2, SH3BGR, VCPIP1, WAS
Nerve	Carom, ELAVL1, GRASP, HTRA1, ITSN1, SH3BGR, VCPIP1, WAS
Pituitary gland	Carom, CCDC104, CCT2, ELAVL1, HSPA5, HTRA1, MAGI1, MSL1, SH3BGR, WASL
Tier 2 "Nearly ready", lack of carom	
Blood	DAPP1, SH3BGR, WAS
Bone	CCDC85B, HTRA1, SH3BGR, WAS
Bone marrow	SH3BGR, WAS
Brain	SH3BGR
Connective tissue	SH3BGR
Embryonic tissue	SH3BGR
Eye	CHKA, GRASP, HTRA1, MSL1, SH3BGR
Heart	CHKA, DAPP1, HTRA1, ITSN1, MSL1, SH3BGR, WAS
Kidney	DAPP1, SH3BGR
Liver	C9ORf78, DAPP1, SH3BGR
Lung	DAPP1, SH3BGR
Muscle	C9ORf78, CASK, CCDC104, DAPP1, HSP90AB1, ITSN1, ITSN2, MAGI1, SH3BGR, VCPIP1, WASL
Ovary	SH3BGR

(Cond..)

Table 2. *(Continued..)*

Tissue	Carom/partner expression
Pancreas	CCDC85B, GRASP, SH3BGR
Spleen	DAPP1, GRASP, SH3BGR, WAS
Testis	CCDC104, CCT2, CHKA, GRASP, SH3BGR, VCP
Thymus	SH3BGR, WAS
Vascular	SH3BGR
Tier 3 "Function privilege", lack of carom & partners	
N/A	N/A
B. Mouse tissues	
Tier 1 "Ready to go", express carom and partners	
Lymph node	Carom, Cct2, Htra1, Itsn1, Itsn2, Msl1, Srp72, Vcpip1
Blood	Carom, Dapp1, Itsn2, Srp72
Eye	Carom, Cask, Chka, Elavl1, Hspa5, Itsn2, Magi1, Msl1, Srp72, Vcpip1
Ovary	Carom, Grasp, Itsn2, Magi1, Srp72, Vcpip1
Spleen	Carom, Cask, Dapp1, Vcpip1
Tier 2 "Nearly ready", lack of carom	
Bone marrow	Vcpip1
Brain	Grasp, Magi1
Extra embryonic tissue	Elavl1
Heart	Grasp
Muscle	Elavl1, Grasp, Htra1, Magi1
Pancreas	Cask, Dapp1, Grasp, Itsn2, Srp72, Wasl
Pituitary gland	Cask, Ccdc104, Cct2, Dapp1, Elavl1, Hspa5, Htra1, Magi1, Msl1, Srp72, Vcp, Wasl
Testis	Chka, Dapp1, Elavl1, Grasp
Thymus	Chka, Dapp1, Itsn2
Tier 3 "Function privilege", lack of carom & partners	
Bone	N/A
Connective tissue	N/A
Embryonic tissue	N/A
Kidney	N/A
Liver	N/A
Lung	N/A

Based on expression patterns of Carom/partner, human and mouse tissues are classified into: Tier 1 tissues "Ready to go" express Carom and partner genes; Tier 2 tissues "Nearly ready" express Carom partner genes but lack of Carom; Tier 3 tissues "Function privilege" lack of Carom and partner gene expression. A. Tissue classification of Carom/partner expression (human tissues). Carom/partner mRNA are highly expressed in lymph node, pituitary gland and nerve of human tissues. B. Tissue classification of Carom/partner expression (mouse tissues). Carom/partner mRNA are highly expressed in blood, eye, lymph node, spleen and ovary of mouse tissues

26 Carom partner proteins have been reported to regulate various cellular functions including endocytosis and vesicle trafficking, cell junctions and cycle, and embryonic stem cell differentiation. Therefore, these functions maybe influenced by signaling via a Carom complex.

Carom was initially identified as an interacting protein of membrane-associated guanylate kinase inverted 1 (MAGI1) by two-hybrid screening (24). As shown in Figure 2, Carom contains an N-terminal F-BAR domain, two C-terminal SH3 domains and a

Table 3. Conditions with altered carom expression (GEO database)

Conditions	Cells	Fold	*P*-value	GDS ID#
Inflammatory conditions				
Hypoxia	Mouse pulmonary ECs	3.8.*	0.0.13238	61
TNF	Mouse microvascular ECs	3.7.	0.0.67240	2773
Endometriosis	Human endometrial ECs	1.6.*	0.0.27669	3060
PPARγ deletion	Mouse aortic ECs	0.5.*	0.0.19506	3440
Hypoxia	Immature MoDCs	1.6.	0.1.40237	2750
VAF347	Immature MoDCs	0.7.	0.0.03019	3193
Quercetin	Human MCs	0.7.	0.1.82310	3676
Atorvastatin	Human FCH MCs	1.0.	0.8.68484	3362
Hypoxia	Human macrophages	2.1.	0.0.59820	2036
IFN-γ	Human macrophages	1.0.	0.9.21205	1365
Hypoxia	Human aortic SMCs	1.0.	0.9.85201	3112
Vitamin D	Human bronchial SMCs	1.2.	0.0.28600	2628
Hypoxia	Human renal epithelial cells	1.0.	0.5.60524	3524
IL-13	Human airway epithelial cells	1.0.	0.9.63000	4981
ox-LDL	Human retinal epithelial cells	1.5.	0.0.14300	2307
Reprogramming conditions				
iPSC generation[1]	Human placental ECs	0.2.*	0.0.00005	3842
Angiogenesis conditions				
Angiopoietin-1	HUVECs	1.3.	0.4.12408	3355
VEGF-A	HUVECs	0.9.	0.4.80878	495
PGF	HUVECs	1.1.	0.5.05246	495

Via GEO database mining, we identified conditions with altered Carom expression in ECs, monocytes, macrophages, SMCs and epithelial cells. The experimental conditions can be classified into inflammatory, reprogramming and angiogenesis. The expression of Carom was significantly altered in hypoxia, endometrial, PPARγ deleted ECs and iPSC generation from ECs. Δ, fold changes of mRNA in treated cells over the expression level of gene in control cells. *Changes ≥1.5. with *P*<0.0.5 was considered as significant increase; Changes ≤ 0.8. with *P*<0.0.5 was considered as significant decrease. [1], human placental ECs was reprogrammed to iPSC by co-infection of retroviral(pMXs)-OCT3/4, SOX2, KLF4 and c-MYC. Abbreviations: FCH, familial combined hyperlipidemia; HUVECs, Human umbilical vein endothelial cells; MoDCs, monocyte-derived dendritic cells; PGF, placental growth factor; PPARγ, peroxisome proliferator-activated receptor gamma; SMCs, smooth muscle cells; TNF, tumor necrosis factor; VEGF-A, vascular endothelial growth factor A. For other abbreviations, refer to Figure 1

PSD-95/Dlg-A/ZO-1 (PDZ) binding motif. It binds to the fifth PDZ domain of MAGI1 via its PDZ binding motif. It was reported that Carom can also bind to calcium/calmodulin-dependent serine protein kinase (CASK) protein. CASK has one calmodulin kinase (CAMK) domain, one PDZ domain, one SH3 domain and one guanylate kinase (GK) domain. Carom binds to CASK via its C-terminal region. Both CASK and MAGI1 belong to membrane-associated guanylate kinases (MAGUK) family, which interact with partners to regulate cell junctions (25). It is suggested that CASK can compete with MAGI1 to complex with Carom and is a dominant regulator for cell junction in polarized epithelial cells (24).

4.2. Carom and 26 partners are differentially expressed in human and mouse tissues

We established tissue mRNA expression profiles of Carom and 26 partners in 21 human and 20 mouse tissues by EST database mining (Figure 3). Confidence intervals were generated based on mRNA expression levels of 3 housekeeping genes (PTTG1IP, PKM2, and HNRNPK), which have relatively consistent mRNA levels across the selected tissues in both human and mouse.

In Figure 3A, PTTG1IP expression profiles are presented as representative of housekeeping genes. The confidence intervals of the housekeeping gene expression are X ± 2SD = 3.2.4 in human tissues and X ± 2SD = 3.3.2

Table 4. Carom partner changes in inflammatory/reprogramming conditions in ECs (GEO datasets)

Genes	Hypoxia		Endometriosis		PPARγ deletion		iPSC generation	
	Fold∆	*P* value	Fold∆	*P* value	Fold∆	*P* value	Fold∆	*P* value
A. Provide title								
Carom	3.8.*	0.0.13238	1.6.*	0.0.27669	0.5.*	0.0.19506	0.2.*	0.0.00005
C2ORF73	-	-	0.6.	0.3.30876	-	-	-	-
C9ORF78	-	-	1.1.	0.4.16593	-	-	1.7.	0.0.02310
CASK	0.8.	0.4.68860	1.5.	0.1.93253	0.5.*	0.0.10005	1.9.	0.0.00582
CCDC85B	-	-	1.8.	0.2.44447	1.1.	0.5.88658	-	-
CCDC104	-	-	-	-	-	-	-	-
CCDC158	0.4.	0.1.90705	0.5.	0.3.99058	1.2.	0.5.04093	-	-
CCT2	-	-	1.2.	0.2.68208	1.1.	0.3.95650	-	-
CHKA	0.6.	0.1.80578	0.8.	0.5.98553	0.4.*	0.0.03614	7.2.	5.9.3E-07
DAPP1	-	-	1.4.	0.2.72458	1.3.	0.4.11033	-	-
ELAVL1	0.8.	0.4.44665	1.3.	0.1.52146	0.7.	0.1.63026	2.8.	0.0.00025
GRASP	0.7.	0.1.73320	1.3.	0.3.46210	1.1.	0.5.21888	0.0.2*	2.6.9E-09
HSP90AB1	-	-	1.1.	0.4.84935	1.1.	0.7.63708	2.6.	0.0.00060
HSPA5	-	-	1.2.	0.1.69979	1.8.	0.0.65709	0.3.*	0.0.00004
HTRA1	1.2.	0.4.09472	1.1.	0.7.76317	0.6.	0.2.90893	0.1.*	1.2.6E-07
ITSN1	0.9.	0.7.73120	1.7.*	0.0.20809	0.5.*	0.0.48634	1.7.	0.0.01040
ITSN2	0.4.*	0.0.34584	0.5.	0.1.17980	0.5.*	0.0.43780	0.4.*	0.0.00139
MAGI1	2.3.*	0.0.27024	0.7.	0.0.74850	0.4.	0.1.30637	3.3.	0.0.00023
MSL1	-	-	1.1.	0.3.77604	0.7.	0.1.18741	1.3.	0.0.20500
SH3BGR	-	-	1.1.	0.6.17677	0.2.*	0.0.21471	-	-
SRP72	-	-	1.3.	0.1.18539	1.2.	0.3.48976	2.1.	0.0.01580
UBC	-	-	1.1.	0.3.49352	0.4.	0.1.69575	0.8.*	0.0.25100
UBD	0.8.	0.7.74258	-	-	1.4.	0.1.63619	-	-
VCP	-	-	1.3.	0.1.29360	1.0.	0.9.67174	0.8.	0.1.02000
VCPIP1	-	-	1.4.	0.2.12843	0.5.	0.0.71022	-	-
WAS	-	-	1.5.	0.1.19350	2.1.	0.2.90033	-	-
WASL	1.4.	0.2.34384	1.7.	0.1.78621	0.6.	0.0.77387	-	-

B. Paired carom & partner changes in ECs	
Conditions	**Paired carom complex** (carom (∆)-Partner (∆))
Inflammatory	
Hypoxia	Carom(3.8.)-MAGI1(2.3.)
Endometriosis	Carom(1.6.)-ITSN1(1.7.)
PPARγ deletion	Carom(0.5.)-CASK(0.5.), CHKA(0.4.), ITSN1(0.5.), ITSN2(0.5.), SH3BGR(0.2.)
Reprogramming	
iPSC generation	Carom(0.2.)-GRASP(0.0.2), HSPA5(0.3.), HTRA1(0.1.), ITSN2(0.4.), UBC(0.8.)

12 of 26 potential Carom partners identified in Table 1 are presented with changed mRNA levels. In hypoxia conditions, Carom and MAGI1 were both induced. In endometrial ECs, Carom and ITSN1 were induced. In PPARγ deleted ECs, Carom was induced along with CASK, CHKA, ITSN1, ITSN2 and SH3BGR induction. During the reprograming of ECs to iPSC, Carom and GRASP, HSPA5, HTRA1, ITSN2 and UBC were induced. These paired induction suggests a potential functional Carom complex in these conditions. ⊗, fold changes of mRNA in treated cells over the expression level of gene in control cells. *, Changes ≥1.5. with *P*<0.0.5 was considered as significant increase; Changes ≤ 0.8. with *P*<0.0.5 was considered as significant decrease. Abbreviations: refer to Figure 1 and Table 3

in mouse tissues, and not significantly different between species. Lines indicating an upper limit of the confidence interval were put in all bar graphs of tissue profiles to establish the significance of gene expression. mRNA expression levels of Carom and partners higher than the upper limit of the confidence interval of housekeeping genes were considered statistically significant higher expression in tissues.

In human tissues, Carom is highly expressed in the lymph node, pituitary gland and nerves in human tissues (Figure 3B). According to the tissue expression profiles of Carom and 26 partners, the 21 human tissues can be classified into three tiers (Table 2A). Tier 1 tissues are "Ready to go" tissue, which expresses Carom and some of the partner genes. Human lymph node, pituitary gland and nerve belong to this Tier 1 category. Tier 2 tissues are "Nearly ready" which express Carom partner genes but lack of Carom, including heart, lung, vascular, brain, pancreas, liver, blood, bone marrow, spleen and others as detailed in the table. Tier 3 tissues are "Function privilege" and lack both Carom and partner gene expression, and are only identified in mouse but not in human.

In mouse tissues, Carom is highly expressed in blood, eye, lymph node, spleen and ovary in mouse tissues (Figure 3C). The expression patterns of Carom and 26 partners can also be classified into three tiers (Table 2B). Tier 1 "Ready to go" tissues include blood, eye, lymph node, ovary and spleen. Tier 2 "Nearly ready" tissues include brain, heart, and pancreas. Tier 3 "Function privilege" tissues include bone, connective tissue, embryonic tissue, kidney, liver and lung.

4.3. Inflammatory and reprogramming conditions altered Carom expression

As indicated in Table 3, via GEO database mining, Carom mRNA levels were found increased by 3.8. fold and 1.6. fold in hypoxia treated mouse pulmonary endothelial cells (ECs) (GDS ID# 61) and in human endometrial ECs (GDS ID# 3060). It was reduced by 0.5. and 0.2. fold in PPARγ deleted mouse aortic ECs (GDS ID# 3440) and during the reprogramming of human placental ECs to iPSC by co-infection of retroviral (pMXs)-OCT3/4, SOX2, KLF4 and c-MYC (GDS ID# 3842). Carom expression levels were not changed in angiogenesis conditions in ECs, nor in monocytes, macrophages, smooth muscle cells and epithelial cells treated with various conditions.

4.4. Paired Carom partners in inflammatory and reprogramming conditions

We found that Carom was altered in inflammatory and reprogramming conditions, including hypoxia, endometrial, PPARγ depletion and iPSC reprogramming (Table 3). We suspected that Carom complex participates in these processes and continued to examine their potential partners. As shown in Table 4A, we identified 12 partners having paired changes with Carom suggesting these partner proteins may have a paired increasing or decreasing complex relationship with Carom. Carom and MAGI1 were both regulated in hypoxia treated ECs. We identified paired induction of ITSN1 with Carom in endometrial ECs. In contrast, Carom was reduced in PPARγ deleted ECs, which was paired with CASK, CHKA, ITSN1, ITSN2 and SH3BGR reduction. During iPSC reprogramming, Carom was reduced along with GRASP, HSPA5, HTRA1, ITSN2 and UBC reduction (Table 4B).

4.5. Carom complex pathway analysis

We identified 26 signaling pathways, which involve the expression changes of Carom partner proteins using IPA software. These pathways have a high threshold determined by publication frequencies. The Protein ubiquitination pathway, which involves Carom complex proteins UBD, UBC, HSP90AB1, HSPA5, was most frequently reported and has the highest -log (*P* value), followed by adherent junction signaling, ephrin receptor signaling and others as detailed in Figure 4. We further screened for reported Carom signaling regulation in these pathways in above four conditions by GEO analysis (Table 4) and Pubmed literature search.

We presented the Pubmed ID related with reported Carom complex signaling pathway regulation and altered Carom partner in Table 5. We found 10 pathways characterized in hypoxia, endometriosis, PPARγ deletion and iPSC generation conditions. Ephrin receptor signaling, clathrin-mediated endocytosis signaling, eNOS signaling, integrin signaling and actin cytoskeleton signaling were changed in hypoxia treated ECs (PMID ID#: 19834031, 23861904, 24938229, 22353471 and 24144209). UBC change was associated with altered clathrin-mediated endocytosis and wnt/β-catenin signaling in iPSC generation. HSPA5 change was associated with altered endoplasmic reticulum stress in iPSC generation. ITSN1 change was associated with altered axonal guidance signaling in endometrial ECs.

As summarized in Table 6, paired Carom (1.6.-fold) and ITSN1 (1.7.-fold) induction is associated with enhanced axonal guidance signaling and angiogenesis phenotype in endometrial ECs. During iPSC reprogramming, paired Carom (0.2.-fold) and HSPA5 (0.3.-fold) reduction is associated with increased endoplasmic reticulum stress; Paired Carom (0.2.-fold) and UBC (0.8.-fold) reduction is associated with decreased clathrin-mediated endocytosis and increased wnt/β-catenin signaling.

5. DISCUSSION

In this study, we employed a group of combined database mining strategies and reported five major findings: (1) identified 26 Carom partners and established

Table 5. Conditions altered carom signaling and partner expression changes in ECs (IPA/GEO/pubmed)

Potential carom complex signaling (partner name)	Conditions alter carom signaling and partner changes (PMID ID#/changed carom partner, Table 4)			
	Hypoxia	Endometriosis	PPARγ deletion	iPSC generation
Protein ubiquitination pathway (UBD,UBC,HSP90AB1,HSPA5)	-	-	-	-
Adherens junction signaling (MAGI1,WAS,WASL)	-	-	-	-
Ephrin receptor signaling (ITSN1,WAS,WASL)	19834031	-	-	-
Clathrin-mediated endocytosis signaling (UBD,UBC,WAS)	23861904	19189995	-	25036638/UBC↓
Unfolded protein response (VCP,HSPA5)	-	-	-	-
Actin nucleation by ARP-WASP complex (WAS,WASL)	-	-	-	-
Huntington's disease signaling (UBD,UBC,HSPA5)	-	-	-	-
GABA receptor signaling (UBD,UBC)	-	-	-	-
Renal cell carcinoma signaling (UBD,UBC)	-	-	-	-
Ephrin B signaling (ITSN1,ITSN2)	-	-	-	-
Toll-like receptor signaling (UBD,UBC)	-	18596029	23105142	-
Regulation of actin-based motility by Rho (WAS,WASL)	-	-	-	-
Hereditary breast cancer signaling (UBD,UBC)	-	-	-	-
Phosphatidylcholine biosynthesis I (CHKA)	-	-	-	-
Phosphatidylethanolamine biosynthesis II (CHKA)	-	-	-	-
eNOS signaling (HSP90AB1,HSPA5)	24938229	23935397	23208382	-
Aldosterone signaling in epithelial cells (HSP90AB1,HSPA5)	-	-	-	-
Axonal guidance signaling (ITSN1,WAS,WASL)	-	25051436/ITSN1↑	-	-
Cdc42 signaling (WAS,WASL)	-	-	-	-
Wnt/β-catenin signaling (UBD,UBC)	-	-	-	25548277/UBC↓
Leukocyte extravasation signaling (WAS,WASL)	-	-	-	-
Integrin signaling (WAS,WASL)	22353471	12372469	21339703	-
Endoplasmic reticulum stress pathway (HSPA5)	-	19001553	-	25145356/HSPA5↓
Actin cytoskeleton signaling (WAS,WASL)	24144209	-	-	24702998
Signaling by Rho family GTPases (WAS,WASL)	-	-	18209083	-
Glucocorticoid receptor signaling (HSP90AB1,HSPA5)	-	-	-	-

Altered Carom signalings or partners involved in 26 potential pathways (Figure 4) in conditions identified in Table 3 were analyzed by Pubmed literature search and GEO database mining (Table 4). Altered Carom signaling are described in manuscript cited by Pubmed ID. Carom partner proteins involved in these signaling are bolded and indicated in the table following Pubmed ID. Abbreviations: refer to Figure 1 and Table 2-4

their tissue expression profiles; (2) classified three tiers of tissues for Carom complex signaling readiness status; (3) discovered four Carom inducing conditions (hypoxia, endometriosis, PPARγ deletion and iPSC reprogramming); (4) predicted 26 potential Carom signal pathways; (5) established a working model of Carom signaling.

5.1. Carom and partner proteins are differentially expressed in tissues

We presented in Figure 3, that Carom is differentially expressed in human and mouse tissues. Each tissue has different expression pattern for Carom and partner proteins. This data suggests that Carom signaling can be differentially regulated and that the readiness of Carom signaling varies in individual tissues. We classified the analyzed 21 human tissues and 20 mouse tissues into three tiers (Table 2). We anticipate that Carom complex signaling is essential in Tier 1 "ready to go" tissue, in which Carom signal is essential and its expression including the partners is ready in normal condition. The Tier 2 tissues require the induction of Carom as it contains only the complementary partners but lack of Carom. It may take longer time to overcome

Table 6. Predicted function of carom complex signaling in Ecs

Conditions	Paired carom complex (carom (Δ)-partner (Δ)	Signal pathways	Functions	PMID ID#
Endometriosis				
	Carom (1.6.)-ITSN1 (1.7.)	Axonal guidance signaling ↑	Angiogenesis ↑	25051436
Reprogramming				
	Carom (0.2.)-HSPA5 (0.3.)	Endoplasmic reticulum stress ↑	iPSC generation ↑	25145356
	Carom (0.2.)-UBC (0.8.)	Clathrin-mediated endocytosis ↓	iPSC generation ↑	25036638
		Wnt/β-catenin signaling ↑	iPSC generation ↑	18682236

Functional implications of Carom complex is evaluated based on identified paired Carom & partner changes (Table 4) and signaling (Figure 4), and relevant functional change reported (Pubmed ID). Endometriosis condition may promote Carom(1.6.)-ITSN1(1.7.) complex, leading to angiogenesis via the activation of axonal guidance signaling. During iPSC reprograming, Carom(0.2.)-HSPA5(0.3.) and Carom(0.2.)-UBC(0.8.) complexes may be suppressed, leading to iPSC generation via increased endoplasmic reticulum stress, reduced Clathrin-mediated endocytosis and activated wnt/β-catenin signaling, respectively. Δ, fold changes of mRNA in treated cells over the expression level of gene in control cells. Abbreviations: refer to Figure 1 and Table 2-5

a threshold in Tier 2 tissues than in the Tier 1 tissues to activate Carom complex signaling. Because neither Carom, nor its partners are expressed in Tier 3 tissues in human, suggesting that Carom systems may not be required in these tissues in human.

It is noticed that some tissues are classified differently in human and mice, except for lymph node. We found that lymph node is the only Tier 1 tissue in both human and mouse, suggesting that Carom complex signaling may be equally essential in both human and mouse for immunological processes. By using GEO database mining, there is limited information of Carom in lymph node. Recently, the development of lymph node has been reported to be dependent on EC-restricted lymphotoxin-β receptor signaling (26). Thus, it is possible that Carom complex initiates signaling pathways in ECs to regulate the development of lymph node. Interestingly, pituitary gland is a Tier 1 tissue in humans but not in mice. It is likely that Carom signaling is more important in humans than in mice for development and hormone production control. Apparently, most of the human tissue are Tier 2 tissues, including blood, eye, ovary and spleen. Carom signaling regulation may be sensitive in most of the human tissues to initiate its downstream functions.

It is worth pointing out that tissue expression profile was determined by database mining approach utilizing the NCBI Gene database and EST database in which mRNA levels were calculated based on the copy number of EST cDNA clone sequencing, as we discussed previously (9-18). We believe that the EST database is reliable for gene mRNA expression in normal tissues and has significant advantages over traditional approaches, such as Northern blots and RT-PCR analysis. Therefore, the expression patterns of Carom and 26 partners are experimentally based and precise.

5.2. Expression of Carom complex is altered in inflammatory and reprogramming conditions

Via GEO database, we identified 12 partner proteins having paired changes with Carom in four different conditions (hypoxia, endometriosis, PPARγ deletion and iPSC reprogramming) treated ECs (Table 3&4). Hypoxia and PPARγ deletion induce inflammatory injuries in ECs, while endometriosis presents with high level of pro-inflammatory cytokines for stimulating ECs (27-29). Accordingly, hypoxia, endometriosis, PPARγ deletion were classified into inflammatory condition. We believe that Carom complex signaling may be regulated and mediate some functional changes in inflammatory and iPSC reprogramming conditions.

Among the inflammatory condition, Carom-MAGI1 pair was induced by hypoxia in ECs and Carom-ITSN1 pair was induced in endometrial ECs. Because hypoxia can induce angiogenesis in ECs via increasing expression of VEGF receptor (30) and endometriosis is an angiogenesis pathology by enhancing secretion of VEGF in endometriotic lesion (31), induced Carom signal may be relevant to pathological angiogenesis. Although Carom expression levels are not changed in angiogenesis conditions in ECs on GEO database mining results, it is possible that Carom is associated with pathological angiogenesis in disease. The pathological connection of Carom-CASK, Carom-CHKA, Carom-ITSN1, Carom-ITSN2 and Carom-SH3BGR pair reduction in PPARγ deleted ECs remains unclear and needs to be established in future studies. It is possible that Carom signaling can prevent iPSC reprogramming as a reduced Carom- signaling may be involved in iPSC reprogramming as Carom-GRASP, Carom-HSPA5, Carom-HTRA1, Carom-ITSN2 and Carom-UBC were reduced in these conditions. These partner proteins may have increased or decreased complex relationship with Carom, and may mediate

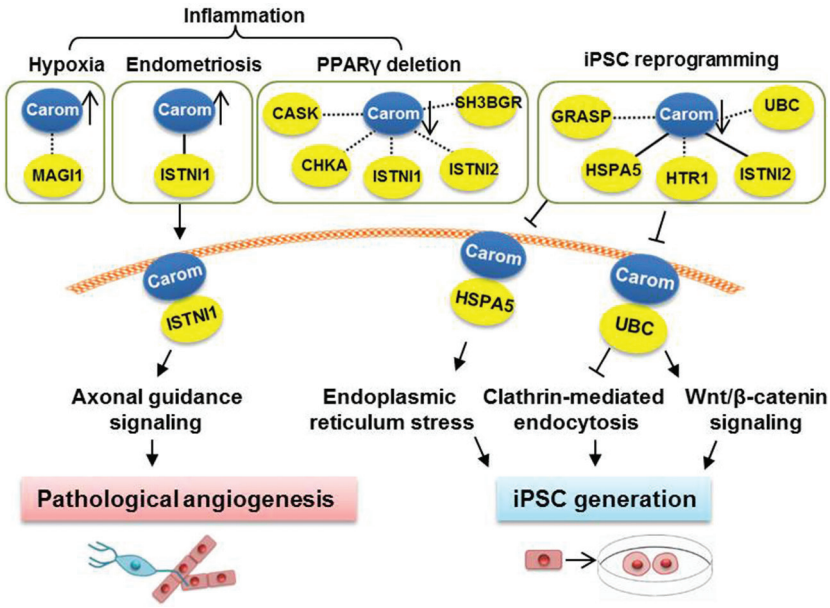

Figure 5. Working model of Carom complex-angiogenesis/iPSC generation in EC. In endometriosis condition, Carom can bind to ITSN1 to activate axonal guidance signaling, leading to pathological angiogenesis. Carom can be induced in hypoxia condition to form complex with MAGI1 and be suppressed in PPARγ deletion condition to weaken the complex with CASK, CHKA, ITSN1, ITSN2, SH3BGR. In iPSC reprograming condition, Carom can also be reduced to inhibit the formation of complex with HSPA5 and UBC, which leads to endoplasmic reticulum stress and wnt/β-catenin signal activation, and reduces clathrin-mediated endocytosis. Carom complex suppression results in iPSC generation. Solid lines represent final working model concluded in this work. Dashed lines represent predicated complex via GEO database analysis (Table 4).

relevant functional changes in inflammatory and iPSC reprogramming conditions.

The GEO database is relatively newer than the EST database. It contains high-throughput experimental data from functional research assessing conditional gene expression changes (32). It is a powerful tool to search the functional connection of novel proteins. Experimental conditions employed in the GEO database provide relevant pathophysiological connections for novel genes with limited functional research evidence.

5.3. Carom signal pathway, function and working model

Using IPA pathway analysis, we identified 26 Carom complex signal pathways based on Carom partner expression changes reported in the Pubmed and national databases (Figure 4). The combinatory analysis of pathway identification and experimentally confirmed Carom pair changes led us to propose a working model of Carom signaling in Figure 5. We hypothesize that Carom-ITSN1 complex activation may mediate pathological angiogenesis and that Carom-HSPA5 and Carom-UBC complex suppression may prevent iPSC generation.

5.3.1. Carom-ITSN1 complex activation and angiogenesis

We found a paired induction of Carom-ITSN1 in endometrial ECs, which is associated with enhanced axonal guidance signaling and angiogenesis phenotype. Endometriosis is a neurovascular disorder characterized by ectopic growth of endometrial lesion outside the uterus (33) along with enriched nerve bundles and blood vessels. We hypothesize that Carom-ITSN1 pair induction may regulate pathological angiogenesis in endometriosis. As indicated in Figure 4 via IPA analysis, ITSN1 was involved in the regulation of axonal guidance signaling. It is reported that ITSN1 encodes Intersectin-1 protein which regulates synaptic vesicle recycling and induces axonal guidance signaling during the development of nervous system in mouse (34). Axonal guidance molecule is reported to promote angiogenesis and suppress neurogenesis in neurons which are complementary effects in endometriosis lesions (35). Therefore, we propose that Carom-ITSN1 complex induction could promote pathological angiogenesis in endometriosis via activation of axonal guidance signaling.

5.3.2. Carom-HSPA5 and Carom-UBC complex suppression and iPSC generation

We identified a paired reduction of Carom-HSPA5 associated with enhanced endoplasmic reticulum stress and Carom-UBC associated with clathrin-mediated endocytosis inhibition and wnt/β-catenin signaling activation during iPSC generation.

HSPA5 encodes the protein Heat shock protein A5, which is a resident protein in the lumen of the endoplasmic reticulum. As indicated in Figure 4,

HSPA5 is involved in the regulation of endoplasmic reticulum stress. Endoplasmic reticulum stress has been reported to facilitate iPSC generation via inducing vascular endothelial growth factor (VEGF) (36). Thus, we hypothesize that Carom-HSPA5 suppression may promote iPSC generation via activation of endoplasmic reticulum stress. It would be interesting to investigate Carom-HSPA5 complex regulation and VEGF secretion, and its relevance with iPSC generation.

UBC represents an ubiquitin gene and encodes polyubiquitin-C protein, which is involved in the regulation of clathrin-mediated endocytosis and wnt/β-catenin signaling (Figure 4). Clathrin-mediated endocytosis regulates signal transduction, nutrient uptake and neurotransmission at the cell membrane (37). In clathrin-mediated endocytosis, F-BAR protein complexes are essential in regulating the formation of clathrin-coated vesicles (2, 38). It has been shown that transforming growth factor-β (TGF-β) acts as a barrier to iPSC generation via inhibition of mesenchymal-epithelial transition (39, 40). Clathrin-mediated endocytosis can positively regulate TGF-β signaling by recycling TGF-β receptors (41). During iPSC generation, clathrin-specific inhibitor Pitstop2 inhibits TGF-β signaling (42). In the presence of TGF-β inhibitor SB431542, Pitstop2 can not suppress TGF-β signaling or increase iPSC generation. Therefore clathrin-mediated endocytosis is also identified as a barrier of reprogramming iPSC via activation of TGF-β signaling. We identify a suppressed Carom-UBC complex during the generation of iPSC which is associated with clathrin-mediated endocytosis signaling. Thus, Carom-UBC suppression may inhibit clathrin-mediated endocytosis and attenuate its barrier function in promoting iPSC generation.

We propose another possible mechanism for Carom-UBC complex induced iPSC generation based on the connection of UBC and wnt/β-catenin signaling we identified via IPA analysis (Figure 4). One possibility is that Carom-UBC complex signaling may contribute to iPSC generation via wnt/β-catenin induced c-Myc, as c-Myc is a well-established target of wnt/β-catenin pathway (43) and a key inducer of iPSC reprogramming. However, it is also possible that wnt/β-catenin signaling may promote iPSC generation independent from c-Myc as it was found that wnt/β-catenin signaling can promote iPSC generation in the absence of c-Myc retrovirus (44). We anticipate that Carom-UBC complex reduction may promote iPSC generation via activating wnt/β-catenin signaling.

6. CONCLUSIONS

In this study, we identified 26 Carom partners and characterized their tissue expression profiles. We found that Carom complex signaling can be induced by inflammatory and iPSC conditions and predicted 26 potential Carom signal pathways. We propose three Carom complex working models. 1) Carom-ISTN1 complex induction promotes pathological angiogenesis. 2) Carom-HSPA5 and Carom-UBC complexes suppress iPSC generation. Our combined database mining strategy is an important advance in merging EST database, GEO datasbase, IPA signal analysis and PubMed literature reviews to identify protein complex working model. This strategy could predict the functional implications and signal pathways of a novel protein complex. The predicted results provide hints and direction for future studies to investigate the role and mechanism of novel protein complex in pathophysiological process of animal models and human diseases.

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