Hyperactivated Wnt-β-catenin signaling in the absence of sFRP1 and sFRP5 disrupts trophoblast differentiation through repression of Ascl2

Haili Bao1,2,3†, Dong Liu2†, Yingchun Xu2, Yang Sun2, Change Mu2, Yongqin Yu2, Chunping Wang2, Qian Han2, Sanmei Liu4, Han Cai1,2, Fan Liu2, Shuangbo Kong1,2, Wenbo Deng1,2, Bin Cao1,2, Haibin Wang1,2*, Qiang Wang3,4* and Jinhua Lu1,2*

Abstract

Background: Wnt signaling is a critical determinant for the maintenance and differentiation of stem/progenitor cells, including trophoblast stem cells during placentation development. Hyperactivation of Wnt signaling has been shown to be associated with human trophoblast diseases. However, little is known about the impact and underlying mechanisms of excessive Wnt signaling during placental trophoblast development.

Results: In the present work, we observed that two inhibitors of Wnt signaling, secreted frizzled-related proteins 1 and 5 (Sfrp1 and Sfrp5), are highly expressed in the extraembryonic trophoblast suggesting possible roles in early placental development. Sfrp1 and Sfrp5 double knockout mice exhibited disturbed trophoblast differentiation in the placental ectoplacental cone (EPC), which contains the precursors of trophoblast giant cells (TGCs) and spongiotrophoblast cells. In addition, we employed mouse models expressing a truncated β-catenin with exon 3 deletion globally and trophoblast-specifically, as well as trophoblast stem cell lines, and unraveled that hyperactivation of canonical Wnt pathway exhausted the trophoblast precursor cells in the EPC, resulting in the overabundance of giant cells at the expense of spongiotrophoblast cells. Further examination uncovered that hyperactivation of canonical Wnt pathway disturbed trophoblast differentiation in the EPC via repressing Ascl2 expression.

Conclusions: Our investigations provide new insights that the homeostasis of canonical Wnt-β-catenin signaling is essential for EPC trophoblast differentiation during placentation development, which is of high clinical relevance, since aberrant Wnt signaling is often associated with trophoblast-related diseases.

Keywords: Sfrp1 and Sfrp5, Hyperactivation, Canonical Wnt pathway, Trophoblast, Ascl2

* Correspondence: haibin.wang@vip.163.com; wqiang2000@126.com; jinhua888@126.com
† Haili Bao and Dong Liu contributed equally to this work.
1Reproductive Medical Center, The First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian, People’s Republic of China
3State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People’s Republic of China
3Full list of author information is available at the end of the article

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Background
The placenta, forming the fetal-maternal interface, is essential for the survival and growth of the fetus in eutherian mammals [1, 2]. In mice, a mature placenta consists of three trophoblast layers: the outermost giant cell layer, the intermediate spongiotrophoblast layer, and the innermost labyrinth layer. The trophoblast cells of the placenta arise from the outer trophoblast of the blastocyst. After implantation (E4.5), while the mural trophoblast cells stop dividing but keep endoreduplication to form the primary trophoblast giant cells invading into the uterus, the polar trophoblast cells maintain proliferating and form diploid extraembryonic ectoderm (ExE) and ectoplacental cone (EPC), from the outer regions of which more TGCs form. Subsequently, the ExE form the chorion layer which gives rise to the labyrinth including two syncytiotrophoblast layers, while the EPC develops into the spongiotrophoblast layer [1].

The balanced differentiation of various trophoblast cell types is a prerequisite for normal placentation [3, 4]. The achaete-scute complex homolog-like 2 (Ascl2, also Mash2), one member of the basic helix–loop–helix (bHLH) family, has been shown to play a critical role during EPC development. Ascl2 is located in the EPC and diminishes as trophoblast cells differentiate into TGCs. Ablation of Ascl2 led to embryonic lethality owing to defective spongiotrophoblast formation [5]. Moreover, ASCL2 is reported to be expressed in human freshly isolated cytotrophoblasts [6] and proximal column extravillous trophoblasts [7], corresponding to the EPC and/or spongiotrophoblast layer in mice. However, the signals that affect EPC trophoblast differentiation and how they interact with Ascl2 remain largely unknown.

Previous studies have provided evidence that Wnt signaling is a critical player during placentation development [8–15]. Wnt7b null mice died at mid-gestation stage due to defective chorioallantoic fusion [10]. Targeted disruption of Wnt2 caused impaired labyrinth [11]. Fzd5 mutation was reported to disturb placental labyrinth development [12], and our previous work further demonstrated the necessity of canonical Wnt2-Fzd5-Gcm1 (Glial cells missing-1) signaling for chorioallantoic branching and trophoblast syncytialization during placentation [9]. Recently, Wnt signal was shown to be essential for the derivation and maintenance of human trophoblast stem cells (HTSCs) and organoids [16–18]. Although Wnt signaling is essential for normal placentation development, hyperactivation of Wnt signaling are often observed in human trophoblast-related diseases such as complete hydatidiform moles and choriocarcinoma [8, 19]. However, whether and how excessive Wnt signaling affects EPC trophoblast differentiation remains largely unclear.

In the present work, we employed a variety of genetic mouse models and cultured trophoblast cells to demonstrate that hyperactivation of canonical Wnt pathway leads to overabundance of TGCs at the expense of spongiotrophoblast cells, via repressing Ascl2 during EPC trophoblast differentiation, providing direct genetic evidence for the crucial role of suitable canonical Wnt signaling during placentation trophoblast differentiation.

Results
Sfrp1 and Sfrp5 deficiency leads to excessive TGC differentiation and compromised placentation development

Secreted frizzled-related proteins (SFRPs), containing a cysteine-rich domain (CRD) that is 30 to 50% similar in sequence to that of the frizzled protein while lacking the transmembrane domain, serve mainly as extracellular inhibitors of Wnt signaling by directly blocking the interactions between the Wnt and Frizzled receptors [20]. Sequence comparison and phylogenetic analysis reveal that SFRP1, SFRP2, and SFRP5 are closely related [21]. To define the potential functions of these SFRPs during placentation, we firstly examine the expression profile of Sfrp1, Sfrp2, and Sfrp5 during early placentation development. Through whole-mount in situ hybridization, we observed that both Sfrp1 and Sfrp5 were expressed in the extraembryonic tissues at E7.5 and E8.5, except Sfrp2 (Additional file 1: Fig. S1A). Further examination of the placental sections at E7.5-E9.5 showed that the expression of Sfrp1 and Sfrp5 was abundant in extraembryonic trophoblast, including trophoblast cells in the chorion and EPC, as well as the subsequent spongiotrophoblast layer (Additional file 1: Fig. S1B). These findings suggest the potential roles of Sfrp1 and Sfrp5 during early placentation development.

To assess the physiological relevance of Sfrp1 and Sfrp5 during placentation development, we examined the pregnancy outcome of Sfrp1 and Sfrp5 double knockout (dKO) females crossed with dKO males, and observed that the average litter size was significantly reduced, compared with that of the wildtype (WT) intercrosses (4.5 ± 0.3 vs 7.0 ± 0.4, *P < 0.05, Fig. 1a). We subsequently analyzed the stage-specific effect of Sfrp1 and Sfrp5 during pregnancy. As illustrated in Fig. 1b, normal embryo implantation exhibited by blue bands was observed in both dKO and WT mice at E4.5, and the average number of implantation sites in dKO mice was comparable to that of the WT mice (6.2 ± 0.5 vs 7.4 ± 0.4). Moreover, Sfrp1 and Sfrp5 deficiency did not hamper uterine decidualization, displayed by the normal weight and size of implantation sites at E7.5 (21.84 ± 0.47 mg vs 21.01 ± 0.94 mg, Fig. 1c). However, increased rate of embryo degeneration was observed in dKO mice at E11.5, (32.4 ± 4.1% vs 6.0 ± 4.3%, *P < 0.05, Fig. 1d). Further histological examination and immunostaining...
Fig. 1 (See legend on next page.)
Analysis of cytokeratin (CK) marking placental trophoblast cells revealed impaired differentiation of extraembryonic trophoblast lineage with expanded TGCs and reduced spongiotrophoblast layer as well as compact chorion, at E8.5–E9.5 (Fig. 1e, f, and Additional file 1: Fig. S2A). In addition, we performed in situ hybridization to examine the expression of marker genes placental lactogen I (Pl1), specifically expressed in TGCs, and trophoblast-specific protein α (Tpbpα), marking EPC and/or spongiotrophoblast cells. While Tpbpα+ trophoblast cells were decreased, Pl1-expressing trophoblast cells were significantly increased in dKO mice (Fig. 1g). These data reveal that Sfrp1 and Sfrp5 deletion disturbed EPC trophoblast differentiation with excessive TGCs during early placentation.

Sfrp1 and Sfrp5 deletion renders hyperactivation of canonical Wnt signaling and disturbs trophoblast differentiation in the EPC

Since Sfrp1 and Sfrp5 regulate both the canonical and noncanonical Wnt pathway, we firstly detected the activity of canonical Wnt pathway. We observed that the nuclear localization of active-β-catenin, an indicator of canonical Wnt signaling activity, was increased significantly in the EPC and TGCs of dKO placentas at E8.5 (Fig. 2a and Additional file 1: Fig. S2B), which was further confirmed by western blot analysis (Fig. 2b). These results suggest that the absence of Sfrp1 and Sfrp5 enhanced the activity of canonical Wnt signaling. Considering the phenotype of reduced EPC trophoblast cells and increased number of TGC in dKO placentas, we
examined the expression of the key genes essential for trophoblast cell differentiation in the EPC. The genes Ascl2 and Hand1 (heart and neural crest derivatives expressed transcript 1), encode two transcription factors of the basic helix-loop-helix (bHLH) family. Ascl2 is required to maintain the EPC progenitor population, determines the specification of Tphpa-positive trophoblast cells, and inhibits Pl1-expressing giant cell differentiation, while Hand1 has opposing roles and promotes Pl1-expressing TGC differentiation [22]. Ablation of Ascl2 or Hand1 in mice both lead to embryo lethality owing to defective placental development [5, 23, 24]. By in situ hybridization, we found that while Ascl2-positive trophoblast cells were decreased (Fig. 2c), the number of Hand1-expression TGCs were increased significantly (Fig. 2d), in dKO mice at E8.5 and E9.5, similar to that of Ascl2 mutant mice [5]. These findings illustrate that loss of Sfrp1 and Sfrp5 increases the canonical Wnt signaling activity which might disturb the differentiation of trophoblast progenitors in the EPC.

Global stabilization of β-catenin leads to impaired placental development with excessive TGC differentiation

Since Sfrp1 and Sfrp5 deletion led to exaggerated canonical Wnt signaling activity, to further define whether the hyperactivation of canonical Wnt signaling disturbed EPC trophoblast differentiation, we introduced the Ctnnb1^f(EX3)/f(EX3) mouse model, in which the exon 3 of β-catenin gene is flanked by loxp sites, and Cre recombinase-mediated excision would give rise to the expression of a stabilized, constitutively active form of β-catenin resistant to degradation by the GSK3 proteasome pathway [25]. Through crossing the Ctnnb1^f(EX3)/f(EX3) male with Prm-cre (functioning in adult testis) transgenic female, Prm-cre; Ctnnb1^f(EX3)/f(EX3) male mice were generated. When crossing WT females with Prm-cre; Ctnnb1^f(EX3)/f(EX3) males, the Ctnnb1^Δ/Δ conception with stabilized β-catenin and hyperactivated canonical Wnt pathway were generated. In this breeding, the average litter size decreased significantly, and Ctnnb1^Δ/Δ pups failed to be born (Fig. 3a, b) and degenerated before E11.5 (Fig. 3c, d). Moreover, histological examination and immunostaining analysis of CK showed excessive TGCs in the Ctnnb1^Δ/Δ conception at E9.5 (Fig. 3e), with increased activity of Wnt signaling (Additional file 1: Fig. S3), similar to the phenotype of Sfrp1, Sfrp5, and Hand1 in mice both lead to embryo lethality owing to defective placental development [5, 23, 24]. By in situ hybridization, we found that while Ascl2-positive trophoblast cells were decreased (Fig. 2c), the number of Hand1-expression TGCs were increased significantly (Fig. 2d), in dKO mice at E8.5 and E9.5, similar to that of Ascl2 mutant mice [5]. These findings illustrate that loss of Sfrp1 and Sfrp5 increases the canonical Wnt signaling activity which might disturb the differentiation of trophoblast progenitors in the EPC.

Trophoblast-specific stabilization of β-catenin impairs EPC trophoblast differentiation with excessive TGC differentiation

To further verify the contributions of trophoblast-specifically stabilized β-catenin protein, we generated the CYP19-cre; Ctnnb1^f(EX3)/f(EX3) mouse model, in which β-catenin protein is dominant-stabilized specifically in extraembryonic trophoblast cells. When CYP19-cre females were crossed with Ctnnb1^f(EX3)/f(EX3), the average litter size was decreased significantly, and the CYP19-cre; Ctnnb1^f(EX3)/f(EX3) offspring failed to be born (Fig. 4a, b) and degenerated before E11.5 (Fig. 4c–e). Histological analysis and immunostaining analysis of CK exhibited abnormal extraembryonic tissues with

Fig. 3 Global stabilization of β-catenin leads to impaired placental development with excessive TGC differentiation. a Average litter sizes of WT females mated with Ctnnb1^f(EX3)/f(EX3) and Prm-cre; Ctnnb1^f(EX3)/f(EX3) males, respectively. *P < 0.05. b Genotyping of the newborns from WT females mated with Prm-cre; Ctnnb1^f(EX3)/f(EX3) males. c Average embryo degeneration rate of WT females mated with Ctnnb1^f(EX3)/f(EX3) and Prm-cre; Ctnnb1^f(EX3)/f(EX3) males, respectively, at E11.5. *P < 0.05. d Genotyping of the remaining survived embryos from WT female mated with Prm-cre; Ctnnb1^f(EX3)/f(EX3) male mice at E11.5. e HE and CK staining of Ctnnb1^f(EX3)/f(EX3) and Ctnnb1^Δ/Δ placental sections at E9.5. In a-d, numbers within and above the bars indicated the number of pregnant mice and embryos examined, respectively. Images in e are representatives of at least three independent experiments. Al, allantois; Dec, decidua; Cp, chorionic plate; Em, embryo; Sp, spongiotrophoblast; TGC, trophoblast giant cell. Scale bar, 100 μm.
excessive TGCs differentiation in CYP19-cre; Ctnnb1f(ΔEX3)/+ conceptus (Fig. 4f), which might be responsible for the embryo degeneration at mid-gestation and recapitulated the phenotypes of the Sfrp1, 5 dKO and Ctnnb1f(ΔEX3)/+ mice. Moreover, while the number of Ascl2- and Tphpa- positive trophoblast cells decreased, Hand1- and Pl1-expressing TGCs were increased significantly in the CYP19-cre; Ctnnb1f(ΔEX3)/+ placentas at E9.5
cre and increased expression of CYP19 were examined in the expression of genes located in different trophoblast cell types, nuclear and cytoplasmic division. Moreover, the expression from the excessive differentiation of TGCs without repressing these molecules; the downregulated genes were related to cell cycle and cell division (Fig. 5c).

Hyperactivation of canonical Wnt signaling disturbs EPC and TGCs of CYP19-cre; Ctnnb1f(EX3)/+ placentas (Additional file 1: Fig. S5), consistent with the findings in the Sfrp1 and Sfrp5 mutant mice. In summary, trophoblast-specific stabilization of β-catenin induced hyperactivation of canonical Wnt pathway which impairs EPC trophoblast differentiation.

Hyperactivation of canonical Wnt signaling disturbs EPC and TGC differentiation via repressing Ascl2 expression

To dissect the underlying mechanism by which hyperactivated canonical Wnt signaling influences trophoblast differentiation, we preformed RNA-seq on CYP19-cre and CYP19-cre; Ctnnb1f(EX3)/+ placentas at E8.5. 209 upregulated genes and 548 downregulated genes were observed in the CYP19-cre; Ctnnb1f(EX3)/+ placentas (fold change > 1.5, P value < 0.05). Gene ontology (GO) enrichment analysis revealed that the upregulated genes, including Prl2c2, Prl3d1, and Prl7b1, were related to the female reproduction and the regulation of lactation (Fig. 5b), possibly due to the increased number of TGCs expressing and/or secreting these molecules; the downregulated genes were related to cell cycle and cell division (Fig. 5c and Additional file 1: Fig. S6), which might be resulted from the excessive differentiation of TGCs without nuclear and cytoplasmic division. Moreover, the expression of genes located in different trophoblast cell types were examined in the CYP19-cre and CYP19-cre; Ctnnb1f(EX3)/+ placentas (Additional file 2: Table S3), and their expression patterns were consistent with the placental defects (Fig. 5d). In addition, we observed that several Wnt target genes, such as Vegfa, Mmp9, Myc, Ppard, and Gcm1, were upregulated in CYP19-cre; Ctnnb1f(EX3)/+ placentas (Additional file 2: Table S3), which was further confirmed by qRT-PCR analysis (Fig. 5e), indicating the enhanced activity of canonical Wnt signaling.

As a marker of progenitor trophoblast cells in the EPC, Ascl2 also functions as an important transcription factor essential for the maintenance of the EPC, since Ascl2 deletion results in increased number of giant cells at the expense of the EPC layer [5], similar to the phenotypes in our present study. Moreover, Ascl2 was reported to be a target of canonical Wnt signaling in intestinal neoplasia and intestinal stem cells [26, 27]. Indeed, according to our RNA-Seq data, Ascl2 expression was decreased in the placenta with hyperactivated canonical Wnt signaling, which was further confirmed by qRT-PCR analysis (Fig. 5f). Based on the above observations, we speculated that Ascl2 might be a target of canonical Wnt signaling during EPC trophoblast differentiation.

Excessive canonical Wnt signaling activity restrains spongiotrophoblast differentiation through suppressing Ascl2 in cultured trophoblast cells

In order to verify the hypothesis that hyperactivation of canonical Wnt signaling disturb the EPC differentiation via repressing Ascl2 expression, we further employed in vitro cultured trophoblast stem (TS) cell lines, which undergo differentiation in the absence of FGF4 and mitomycin C-treated mouse embryonic fibroblast-conditioned medium (FCM) [28]. Compared with WT TS cells, Sfrp1 and Sfrp5 dKO TS cells showed decreased expression of Ascl2 and Tpbpa during differentiation (Fig. 6a, c), consistent with the in vivo observations. Moreover, the level of active-β-catenin protein (Fig. 6b) and the nuclear localization of active-β-catenin (Fig. 6c) were increased significantly in the absence of Sfrp1 and Sfrp5. TOP-Flash assay further confirmed hyperactivated β-catenin transcription activity in Sfrp1 and Sfrp5 dKO TS cells (Additional file 1: Fig. S7A). To confirm the contributions of hyperactivated canonical Wnt pathway to disturbed trophoblast cell differentiation, we employed CHIR99021 (CHIR), the agonist of canonical Wnt pathway [29]. After treatment with CHIR, the nuclear localization of β-catenin was enhanced (Fig. 6d), and the levels of active-β-catenin protein were increased remarkably (Fig. 6e). Meanwhile, the expression level of Ascl2 and Tpbpa were decreased significantly (Fig. 6d, f). On the contrary, Sfrp1 and Sfrp5 dKO TS cells treated with XAV939, an inhibitor of Wnt pathway, or with Ascl2 overexpression exhibited increased expression of Tpbpa and decreased PI1 expression (Additional file 1: Fig. S7B and C). These findings demonstrate that excessive canonical Wnt signaling represses Ascl2 expression which is responsible for the disturbed trophoblast differentiation.

Generally, upon the activation of canonical Wnt pathway, stabilized β-catenin translocate into the nucleus, where it serves as a coactivator of the TCF/LEF DNA binding factors, to activate the transcription of target genes. In colorectal cancer cells, with the assistance of a neighboring cis-acting lincRNA, TCF4/β-catenin complex was recruited to the Ascl2 enhancer immediately downstream of the Ascl2 locus to drive high-level Ascl2 expression [30]. Moreover, β-catenin bound to enhancers and direct enhancer-promoter looping at mesendodermal (ME) lineage genes in human embryonic stem cells (hESC) [31]. To testify how canonical Wnt signaling regulate Ascl2 expression, we performed
Fig. 5 (See legend on next page.)
chromatin immunoprecipitation and sequencing (ChIP-Seq) to assess the genome-wide occupancy of β-catenin in differentiated TS cells in the presence of CHIR or not. As expected, β-catenin occupied the promoter regions of the known Wnt target genes, such as Axin2 and Gcm1 (Fig. 6g). However, β-catenin exhibited enhanced enrichment at a site about 20 kb upstream of the Ascl2 promoter in the presence of CHIR (Fig. 6h), which was confirmed by ChIP-qPCR analysis (Fig. 6i). These data suggest that hyperactivation of canonical Wnt signaling might repress Ascl2 expression through β-catenin binding to the remote upstream regions of the Ascl2 locus during trophoblast differentiation.

**Discussion**

The canonical Wnt-β-catenin signaling is essential for a variety of biological processes, including embryogenesis, stem cell maintenance and differentiation, even the cell self-renewal of cancer stem cells [32–34]. Loss or hyperactivation of canonical Wnt signaling often leads to disturbed developmental processes or even diseases [32, 35]. We provide herein genetic and molecular evidence that hyperactivation of canonical Wnt signaling disturbs trophoblast differentiation in the EPC via repression Ascl2 expression during placental development. These findings highlight the necessity of suitable Wnt signaling during trophoblast differentiation.

Wnt signaling has been reported to regulate the proliferation and differentiation of stem and progenitor cells, during the processes of both embryonic development and adult tissue homeostasis [34, 36], and aberrant Wnt signaling underlies various human diseases [32]. Human colon cancer with APC mutation, rendering inappropriate β-catenin stabilization and hyperactivated canonical Wnt pathway [37], exhibited a hereditary cancer syndrome named familiar adenomatous polyposis (FAP) [38, 39]. Excessive activation of Wnt signaling led to pathological bone deposition and hardening [32]. As to placental development, previous studies showed that the deletion of several members of Wnt signaling pathway impaired placental development [9–15]. Moreover, Fzd5-mediated canonical Wnt signaling has been proven essential for trophoblast syncytialization in both human and mouse [9]. Recent findings demonstrate the necessity of Wnt signaling for the derivation and maintenance of hTSCs [17] and organoids [16, 18]. However, excessive Wnt signaling disturbs development and functions of placental trophoblast cells. In complete hydatidiform mole (CHM) placenta, elevated expression of nuclear β-catenin in the extravillous trophoblasts (EVT) were observed, indicating that aberrant hyperactivation of Wnt pathway contributes to abnormal invasive trophoblast differentiation and invasion [19]. Moreover, inactivation of negative regulators of Wnt signaling, such as APC and SFRP2, was found in choriocarcinoma cells, suggesting that higher activity of Wnt pathway is involved in the progress and function of trophoblast cancers [40, 41]. On the basis of the findings, we wonder what would occur if the activity of Wnt signaling were elevated abnormally during placental development. In the present study, employing different genetic mouse models (Sfrp1 and Sfrp5 dKO mice, Prm-cre; Ctnnb1f(EX3)/+, global null mice, and Cyp19-cre; Ctnnb1f(EX3)/+ trophoblast-specific null mice) that render hyperactivation of canonical Wnt-β-catenin pathway, we found that increased activity of canonical Wnt pathway promoted the excessive differentiation of TGCs via downregulating Ascl2 expression. In addition, since canonical Wnt activity has been reported to promote trophoblast invasion [42], hyperactivation of canonical Wnt signaling in the differentiated TGCs might abnormally promote the invasive ability of these TGCs, leading to their excessive invasion into the maternal decidua, which may help explain the pathogenesis of human accrete pregnancy and worth further investigation.

In addition, as the inhibitors of Wnt pathway, loss of Sfrp1.5 induced hyperactivation of canonical Wnt signaling which impaired trophoblast differentiation, suggesting that the mechanism that keeps the activity of canonical Wnt signaling in a suitable state to maintain the niche for trophoblast differentiation and TGC development, exists in vivo during normal placentation. In order to maintain the niche, a complicated signaling network is needed to preserve the balance of trophoblast differentiation.

Ascl2 is essential for the maintenance of the EPC during early placental development in mice [5]. Ascl2 knockout and hypomorphic mouse model showed abnormal placentation with decreased EPC layer and/or spongiotrophoblast cell lineage as well as increased number of TGCs [5, 43]. In the present study, we found the similar phenomenon when canonical Wnt signaling...
Fig. 6 Excessive canonical Wnt signaling activity restrains spongiotrophoblast differentiation through suppressing Ascl2 in cultured trophoblast cells. a The expression of Ascl2 and Tpbpα was analyzed by qRT-PCR in WT as well as Sfrp1 and Sfrp5 dKO trophoblast differentiated for D0-D6. N = 3. *P < 0.05. b Western blot analysis of active-β-catenin during WT and dKO trophoblast differentiation. c Immunostaining of β-catenin (green) and ASCL2 (red) in WT and dKO trophoblast cells at D4. Arrowheads indicated nucleus-located β-catenin. DAPI-labeled nuclei in blue. d, e Immunostaining of β-catenin (green) and ASCL2 (red), and western blot analysis of active-β-catenin in the presence or absence of CHIR at D4. Arrowheads indicated nucleus-located β-catenin. DAPI-labeled nuclei in blue. f The expression of Ascl2 and Tpbpα was detected by qRT-PCR in the presence of CHIR or not, at D4. N = 3. *P < 0.05. g–i After treatment with CHIR for 36 h, ChIP-seq showing β-catenin enrichment at Wnt targeted gene loci (Axin2 and Gcm1) (g), and the locus about 20 kb upstream of Ascl2 promoter (h), which is confirmed by ChIP-qPCR analysis (i). N = 3. *P < 0.05. β-actin served as the internal control in b, e. Values are normalized by Gapdh expression level and indicated as means ± SD in a, f, i. Images in c, d are representatives of at least three independent experiments. Scale bar, 100 μm.
was hyperactivated and Ascl2 expression was repressed. However, little is known concerning how canonical Wnt signaling regulates Ascl2 expression. In most cases, the canonical Wnt-β-catenin signaling stimulates the transcription of target genes, such as c-Myc [44], Axin2 [45] and Gcm1 [9, 15], through promoter binding by the transcriptional activator complex containing LEF/TCF and β-catenin. In the present work, we observed that β-catenin exhibited enhanced enrichment at the site about 20 kb upstream of Ascl2 locus upon CHIR treatment. However, TCF/LEF might be not required for β-catenin-mediated Ascl2 suppression (Additional file 1: Fig. S8). Since β-catenin has been reported to bind to the downstream or upstream enhancer to promote the expression of its target genes [30, 31], we speculated that excessive canonical Wnt signaling might regulate Ascl2 expression in a similar manner, but to repress Ascl2 expression. Moreover, recent findings have demonstrated that canonical Wnt pathway inhibits the expression of its target genes, including the tumor suppressor 15-prostaglandin dehydrogenase [46], RANKL [47] and E-cadherin [48]. However, the exact mechanism via which canonical Wnt signaling represses gene expression requires further exploration.

The chorionic development was also impaired, even though the expression of Gcm1, essential for chorionic trophoblast differentiation [49] and regulated by canonical Wnt signaling [9], was increased upon the hyperactivation of canonical Wnt pathway (Fig. 5e). One possibility is that while canonical Wnt signaling is essential for chorionic trophoblast differentiation, hyperactivation of canonical Wnt signaling which increases Gcm1 expression, is not necessarily beneficial for terminal differentiation of chorionic trophoblast. This could be explained by the fact that Gcm1 overexpression just induces a rapid arrest of trophoblast proliferation and restricts their differentiation fate towards syncytiotrophoblast cells, but is not sufficient to render cell shape changes or cell–cell fusion during subsequent terminal differentiation and syncytium formation. Another possibility is that the disturbed EPC differentiation with reduced Ascl2 expression resulting from hyperactivated canonical Wnt signaling, led to defective chorionic trophoblast differentiation, since the mouse models with Ascl2 deletion [5] or reduced Ascl2 expression [50] also displayed disturbed chorionic/labyrinth development. Even though it has been shown that Ascl2-expressing trophoblast cells was not required for labyrinth development [51], this work is not imprecisely, since the wild-type trophoblast could provide the labyrinth layer with Ascl2-expressing trophoblast cells. In addition, the EPC/spongiotrophoblast layer might functions as structural support for the chorionic/labyrinth development, since Ascl2-expressing trophoblast cells are also present in the chorion and subsequent labyrinth layer.

Conclusions

In summary, hyper-activation of the canonical Wnt signaling, achieved by either the deletion of Sfrp1,5 or the expression of a stabilized β-catenin globally and trophoblast-specifically, resulted in impaired EPC trophoblast differentiation and excessive TGC expansion. Further exploration using the trophoblast stem cell line uncovered that hyper-activated Wnt pathway led to the repression of Ascl2, which is important for trophoblast differentiation. This study provides genetic and molecular evidence that appropriate canonical Wnt pathway is crucial for EPC trophoblast differentiation, which is of high clinical significance since there is a strong correlation between abnormal Wnt signaling and human trophoblast diseases [8].

Materials and methods

Animals and tissue collection

Ctnnb1β(Ex3)/β(Ex3) mice, Sfrp1 and Sfrp5 double mutant mice, and Cyp19-cre transgenic mice were generated as previously described [25, 52–54]. Prm-cre transgenic mice were obtained from Jackson Laboratory. Ctnnb1β(Ex3)/β(Ex3) mice were mated with Prm-cre mice to get mice with global hyperactivation of the canonical Wnt pathway. Ctnnb1β(Ex3)/β(Ex3) mice were mated with Cyp19-cre transgenic mice to get trophoblast-specific hyperactivation of canonical Wnt signaling. Eight-week females were mated with fertile males to induce pregnancy and the day when vaginal plugs were seen was considered as embryonic day 0.5 (E0.5). Pregnant females were sacrificed and implantation sites were weighted, frozen, or fixed in 10% neutral buffered formalin. Tail genotyping of the embryos and newborns were determined by PCR.

Histological analysis and immunostaining

Histological and immunostaining analysis were performed as described previously [9]. In brief, dissected implantation sites were fixed in 10% neutral buffered formalin at room temperature overnight. Tissues underwent dehydration using graded ethanol, vitrification by dimethylbenzene and were embedded in paraffin, and 5 μm transverse sections were used for hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), and immunofluorescence (IF). For frozen tissue, 10 μm transverse sections were used for immunofluorescence (IF). Antibodies used for immunohistochemistry (IHC) and immunofluorescence (IF) include: cytokeratin (DAKO, Z0622, 1:200), active β-catenin (CST, #8814, 1:1000), β-catenin (Abcam, ab6302, 1:1000), Plf (Santa Cruz, sc47347, 1:200), and Pcdh12 (MAB7926, 1:200).

In situ hybridization

In situ hybridization with isotopes-labeled antisense RNA probes was performed on cryosections (10 μm) as
previously described [55]. Whole-mount in situ hybridization with digoxigenin (DIG)-labeled antisense RNA probes was conducted using standard procedures. The primers for probe production are listed in Additional file 1: Table S1.

Trophoblast stem cell derivation, culture, and differentiation

Sfrp1 and Sfrp 5 dKO as well as WT TS cells were derived from E3.5 mouse blastocysts as described previously [28]. Briefly, blastocysts were obtained from E3.5 uterus, and transferred to four-well tissue culture dish containing the mitomycin C-treated MEF feeders in TS medium+F4H (RPMI 1640 (Gibco, 31870082) containing 20% FBS (Gibco, 16000-044), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, 2 mM L-glutamine, 25 ng/ml FGF4 (Peprotech, 100-31), and 1 mg/ml Heparin (Sigma, 2,608,411)) and cultured at 37 °C, 5% CO2. Then, the blastocysts hatched from zona pellucida and attached to the wells to form outgrowth. After disaggregation of the blastocyst outgrowth, feed cells with 70%FCM + 1.5xF4H (30% TS medium, 70% mouse fibroblast-conditioned medium, 1.5x FGF4/Heparin) for early passages and with 70%FCM + 1xF4H for maintenance. For TS cell differentiation, TS cell medium was used without the supplementation of FGF4, heparin, and FCM. For the treatment of the blastocyst outgrowth, feed cells with 70%FCM + 1.5xF4H (30% TS medium, 70% mouse fibroblast-conditioned medium, 1.5x FGF4/Heparin) for early passages and with 70%FCM + 1xF4H for maintenance. For TS cell differentiation, TS cell medium was used without the supplementation of FGF4, heparin, and FCM. For the treatment of the blastocyst outgrowth, feed cells with 70%FCM + 1.5xF4H (30% TS medium, 70% mouse fibroblast-conditioned medium, 1.5x FGF4/Heparin) for early passages and with 70%FCM + 1xF4H for maintenance. For TS cell differentiation, TS cell medium was used without the supplementation of FGF4, heparin, and FCM.

Quantitative real-time PCR

RNA extraction and quantitative RT-PCR was performed as described [9]. Briefly, cells were directly lysed in RNAiso plus (TAKARA, 9109) after pumping out the medium. RNA was extracted in the upper layer after chloroform added and centrifuged. Purified RNA could be obtained after precipitation with isopropanol and washing with ethanol. cDNA was reverse transcribed with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TAKA RA, RR047A) according to the manufacturer’s instructions. qRT-PCR were performed with TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (TAKARA, RRS20A). All assays were performed at least three times. The primers for real-time PCR are all listed in Additional file 1: Table S1.

Western blotting

Protein extraction and Western blotting were performed as described previously [9]. Antibodies used for western blotting include active β-catenin (CST, #8814, 1:1000), β-catenin (Abcam, ab6302, 1:1000), and β-actin (Bioworld, AP0063, 1:5000).

RNA-seq and data analysis

RNA from Cyp19-cre and Cyp19-cre; CtmmbF(Ex3)/+ placentas at E8.5 were extracted using RNeasy Micro Kit (QIAGEN, 74004) according to the manufacturer’s instructions. Purified RNA was prepared using TrueSeq® RNA Sample Preparation V2 (Illumina, RS-122-2001) and subjected to 50-bp single-end sequencing with a BGISEQ-500 sequencer. RNA-seq raw data were initially filtered to obtain clean data after quality control. Clean data were aligned to the mouse genome (mm10) by HISAT2. Raw counts for each gene were calculated by EdgeR. DEGs were defined as genes with P value less than 0.05 and fold change larger than 1.5.

ChIP-Seq and ChIP-qPCR

ChIP was performed according to the reported previously [56]. Briefly, about 2 × 10^6 TS cells were cross-linked with 16% formaldehyde (Cell Signaling Technology, 12,606) at final concentration of 1% at room temperature for 10 min and quenched with 1/10 volume of 1.25 M glycine for 15 min on ice. Cell lysate in lysis buffer III were sonicated using Bioruptor pico (Diagenode) and then incubated with 4 μg non-phospho (active) β-catenin antibody (Cell Signaling Technology, 8814) overnight at 4°C with rotation. Immunoprecipitated complexes were collected with 15 μl Protein A Dynabeads (Invitrogen, 10006D) for 1 h at 4°C with rotation. Subsequently, beads were washed sequentially once with low-salt buffer, twice with high-salt buffer, once with LiCl buffer, twice with TE, and then eluted in 400 μl of elution buffer for 30 min at 65°C with shaking. The eluates were incubation at 65°C for 8 h to reverse the cross-linking. Next, eluates were treated with proteinase K for 1 h at 55°C and then RNase A for 30 min at 37°C before DNA was extracted and purified. The ChIP libraries were prepared according to the instruction manual using KAPA DNA HyperPrep Kits (Roche, KK8502) and then run on the Illumina sequencer Hiseq-Xten PE150. Primers for qPCR were listed in Additional file 1: Table S1.

ChIP-Seq analysis

The primary analysis of ChIP-Seq datasets were performed by using Illumina’s Genome Analysis pipeline. The sequencing reads were aligned to the mouse genome (mm10) by HISAT2. Only uniquely aligned reads were kept. MACS2 was applied for peak call using default parameters.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism 8 software. In data that was normally distributed, Student’s t test was performed to determine the significance of a difference between two groups. When comparing the means of more than two groups, a one-way ANOVA was used. Data were presented as means ± SEM in all experiments unless otherwise indicated. *P < 0.05 was considered to indicate a significant result.
Additional file 1: Figure S1. The expression of Sfp1, Sfp2 and Sfp5 during early placentation development. A The expression of Sfp1, Sfp2, and Sfp5 was analyzed by whole-mount in situ hybridization at E7.5 and E8.5. B The expression of Sfp1 and Sfp5 was detected by in situ hybridization at E7.5-E9.5. The signals were pink. Images in (A) and (B) are representatives of at least two independent experiments. Figure S2. Statistical description of the phenotype of the Sfp1 and Sfp5 dKO placenta. A Statistical analysis of spongiotrophoblast thickness and TGCs number in WT and dKO placenta on E9.5. B Quantification of active-β-catenin signal intensity in Fig. 2a. *, P < 0.05. Figure S3. Nuclear localization of active-β-catenin increased in the placenta of Ctnnb1+/–/+ and Cmbh+/–/+ conceptus. A Immunohistochemistry of active-β-catenin in Ctnnb1+/–/+ and Cmbh+/–/+ placenta on E9.5. B Quantification of active-β-catenin signal intensity in (A). *, P < 0.05. Figure S4. Global stabilization of β-catenin leads to impaired trophoblast development and embryonic lethality. HE and CK staining of the sections of Ctnnb1+/–/+ and Cmbh+/–/+ conceptus on E7.5. Images are representatives of at least three independent experiments. Dec, decidua; EPC, ectoplacental cone. Em, embryo; TGC, trophoblast giant cell. Figure S5. Trophoblast-specific stabilization of β-catenin induces hyper-activation of canonical Wnt pathway. A The localization of active-β-catenin was revealed by immunostaining at E7.5. Cy3-labeled active β-catenin in red, DAPI-labeled nuclei in blue. Images are representatives of at least three independent experiments. B Quantification of active-β-catenin signal intensity in (A). *, P < 0.05. Figure S6. Decreased genes with trophoblast-specific stabilization of β-catenin are related to cell cycles. KEGG analysis of the decreased genes (A, B) between Cyp19+/- and Cyp19-/-; Ctnnb1+/–/+ and Ctnnb1+/–/+ placenta (Fold change >1.5). Venn diagram (C). Figure S7. β-catenin was revealed by immunostaining at E7.5. Cy3-labeled active β-catenin in red, DAPI-labeled nuclei in blue. Images are representatives of at least three independent experiments. Figure S8. TCF/LEF might not be required for β-catenin-mediated Ascl2 suppression. A The expression of Ascl2 in WT TS cells treated with indicated conditions. IRF3, a small molecule that abrogates β-catenin-mediated TCF interaction. *, P < 0.05. B The top 13 conserved transcription factors binding motif at the β-catenin binding site, 20 kb upstream of the Ascl2 gene. Table S1. Primers Information. Additional file 2: Table S2. Differentially expressed genes (DEG) between the Cyp19+/- and Cyp19-/-; Ctnnb1+/–/+ placenta (fold change > 1.5). Table S3. Genes expressed in the Cyp19+/- and Cyp19-/-; Ctnnb1+/–/+ placenta.

Abbreviations
Ascl2: achaete-scute complex homolog-like 2 (also known as Mash2); EPC: Ectoplacental cone; ExE: Extraembryonic ectoderm; Gcm1: Glial cells missing-1; Hand: Heart and neural crest derivatives expressed transcript 1; TGC: Trophoblast giant cell

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Authors’ contributions
L.J., W.Q., and W.H. designed the research; B.H., L.D., X.Y., S.Y., M.C., Y.Y., W.C., H.Q., L.S., and C.H. performed the research; L.F., D.W., C.B., and K.S. analyzed the data; L.J., W.Q., B.H., and W.H. wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
All sequencing data including RNA-seq and ChIP-seq data have been deposited in the Gene Expression Omnibus (GEO) database with the accession codes GSE146433 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146433) and GSE146432 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146432) [57], respectively. If any additional information and materials are needed, they will be available upon request from the corresponding authors.

Ethics approval and consent to participate
Mice were housed in the Animal Care Facility of Xiamen University, and animal experiments mentioned in the manuscript have been conducted according to the guidelines for the care and use of laboratory animals.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Author details
1Reproductive Medical Center, The First Affiliated Hospital of Xiamen University, Xiamen 361003, Fujian, People’s Republic of China. 2Fujian Provincial Key Laboratory of Reproductive Health Research, School of Medicine, Xiamen University, Xiamen 361102, Fujian, People’s Republic of China. 3State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People’s Republic of China. 4Department of Surgery, The Ohio State University Wexner Medical Center, Ohio 43210 Columbus, USA.

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