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Transcriptome Analysis of PPARγ Target Genes Reveals the Involvement of Lysyl Oxidase in Human Placental Cytotrophoblast Invasion

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Abstract

Human placental development is characterized by invasion of extravillous cytotrophoblasts (EVCTs) into the uterine wall during the first trimester of pregnancy. Peroxisome proliferator-activated receptor γ (PPARγ) plays a major role in placental development, and activation of PPARγ by its agonists results in inhibition of EVCT invasion in vitro. To identify PPARγ target genes, microarray analysis was performed using GeneChip technology on EVCT primary cultures obtained from first-trimester human placentas. Gene expression was compared in EVCTs treated with the PPARγ agonist rosiglitazone versus control. A total of 139 differentially regulated genes were identified, and changes in the expression of the following 8 genes were confirmed by reverse transcription-quantitative polymerase chain reaction: a disintegrin and metalloproteinase domain12 (ADAM12), connexin 43 (CX43), deleted in liver cancer 1 (DLC1), dipeptidyl peptidase 4 (DPP4), heme oxygenase 1 (HMOX-1), lysyl oxidase (LOX), plasminogen activator inhibitor 1 (PAI-1) and PPARγ. Among the upregulated genes, lysyl oxidase (LOX) was further analyzed. In the LOX family, only LOX, LOXL1 and LOXL2 mRNA expression was significantly upregulated in rosiglitazone-treated EVCTs. RNA and protein expression of the subfamily members LOX, LOXL1 and LOXL2 were analyzed by absolute RT-qPCR and western blotting, and localized by immunohistochemistry and immunofluorescence-confocal microscopy. LOX protein was immunodetected in the EVCT cytoplasm, while LOXL1 was found in the nucleus and nucleolus. No signal was detected for LOXL2 protein. Specific inhibition of LOX activity by β-aminopropionitrile in cell invasion assays led to an increase in EVCT invasiveness. These results suggest that LOX, LOXL1 and LOXL2 are downstream PPARγ targets and that LOX activity is a negative regulator of trophoblastic cell invasion.

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Introduction

Human placental development relies on trophoblast differentiation along two pathways. Villous cytotrophoblasts (VCTs) fuse to form a syncytiotrophoblast (ST) involved in placental exchanges and endocrine function, while extravillous trophoblasts (EVCTs) anchor the chorionic villi in the maternal uterus. A subpopulation of EVCTs ceases to divide and invades the uterine wall as far as the innermost third of the myometrium and the maternal spiral arteries [1,2] (Figure 1). This invasion coincides with remodeling of arterial walls, resulting in low-resistance blood vessels providing optimal maternal-fetal exchanges. Limited maternal perfusion of the intervillous space, together with histiotrophic nutrition from uterine glands, protects the fetus from high oxygen tension during early stages of differentiation [3,4]. Trophoblast plugging of the maternal spiral arteries between 6 to 8 weeks of gestation is gradually eliminated between 8 to 12 weeks of gestation, leading to increases in intervillous oxygen tension and placental expression of anti-oxidant enzymes [5,6]. This physiological trophoblastic cell invasion process is tightly controlled during the first trimester and is required for placental development and normal pregnancy outcome. Indeed, impaired trophoblast invasion has been implicated in gestational pathologies such as fetal growth restriction and preeclampsia [7,8,9].

To study early human placental development and the regulation of the trophoblastic cell invasion process, we have developed an in vitro invasion model using non proliferative and highly invasive EVCT primary cells isolated from first-trimester human chorionic villi cultured on MatrigelTM [10,11,12]. These purified primary EVCTs express in vitro the specific markers of human invasive EVCTs described in situ, namely cytokeratin 7 [13,14,15], human leukocyte antigen-G [16], human placental lactogen [17], c-erbB2 [18] and the alpha 5 subunit of the fibronectin receptor 581 [19]. The limiting step of this unique human primary culture model is
the low number of EVCTs that can be isolated from early first-trimester placental tissue (8–9 weeks of amenorrhea, WA).

Using this model, we have previously shown that peroxisome proliferator-activated receptor γ (PPARγ) activation by the synthetic and specific agonist rosiglitazone [20] inhibits EVCT invasiveness in a concentration-dependent manner, reaching statistically significant 50% inhibition at a concentration of 1 μM [12]. PPARγ, a member of the ligand-activated nuclear receptor superfamily, controls the expression of many genes involved in metabolism, cell differentiation and tumorigenesis. DNA binding of PPARγ to its response element PPRE (composed of a direct repeat of the core hexanucleotide motif AGGTCA with one intervening base named DR1) requires heterodimerization with another nuclear receptor, the retinoid X receptor (RXR) (for review see [21]). In mice, PPARγ gene inactivation results in abnormal placental development, with defects in trophoblast differentiation and vascular processes leading to embryonic lethality at E10 [22,23]. However, PPARγ expression in trophoblasts is sufficient to rescue PPARγ/− embryonic lethality [24,25]. These studies demonstrated that trophoblastic expression of PPARγ is essential for implantation and for the formation of a functional placenta in mice. In the human placenta, PPARγ is exclusively located in the nuclei of villous trophoblasts throughout pregnancy and, from first trimester placentas, in extravillous trophoblasts. PPARγ is thus a trophoblast-specific marker that can be immunodetected in cytokeratin 7-positive VCT, ST and EVCT throughout differentiation [12,26,27,28].

Here we used our in vitro model of invasive primary EVCTs to identify genes involved in PPARγ-mediated trophoblast invasion, based on a transcriptomic approach. Expression of numerous genes was modulated by rosiglitazone treatment of EVCTs isolated from 8–9 WA placentas. To confirm the transcriptomic results, we used RT-qPCR to analyze eight PPARγ target genes [12,26,27,28] that were the most strongly modified and/or were potentially involved in EVCT invasion, such as dipeptidyl peptidase 4 (DPP4), heme oxygenase 1 (HO-1, HMOX1), connexin 43 (CX43, GJA1), plasminogen activator inhibitor 1 (PAI-1, SERPINE1) and lysyl oxidase (LOX, EC 1.4.3.13).

LOX is a copper-dependent monooxygenase known to catalyze the formation of covalent cross-links of lysine residues within components of the extracellular matrix (ECM), including fibrillar collagen and elastin [29]. Molecular oxygen is necessary to complete the catalytic cycle of LOX, with the release of hydrogen peroxide and ammonia [29]. LOX expression and activity are both dependent on oxygen levels [30]. Like LOX, the four members of the LOX-like family (LOXL1, LOXL2, LOXL3 and LOXL4) display catalytic activity and have both ECM and cellular functions [31]. They all contain a signal peptide and a putative PPARγ response element (Genomatix Software GmbH, http://www.genomatix.de, accessed 2013). These five isoforms can be divided into two subgroups according to their sequence, structure and processing. LOXL2, LOXL3 and LOXL4 contain four scavenger receptor cysteine-rich (SRCR) domains, but little is known of their processing. LOX and LOXL1 are largely homologous, do not contain a SRCR domain, and are secreted as proproteins; this is the subfamily we studied in invasive EVCTs – LOXL2 was also considered.

Among the trophoblastic PPARγ target genes revealed by our transcriptomic approach, we focused on the expression and location of the LOX, LOXL1 and LOXL2 isoforms in early first-trimester human placental tissues in situ and in primary invasive EVCTs in vitro. The role of these isoforms in EVCT invasion was addressed in experiments using their specific catalytic inhibitor β-aminopropionitrile (BAPN) [31] and our primary culture model.

**Results**

**Gene expression profiling of rosiglitazone-treated trophoblasts**

Gene profiling of EVCTs isolated from first-trimester human placenta, with comparisons of rosiglitazone-treated and control cells from the same placenta, was performed with an Affymetrix
GeneChip analyzing 14,500 genes with 22,000 probe sets. The microarray data are available in the gene expression omnibus database (http://www.ncbi.nlm.nih.gov/geo, 2012 GSE20426). Mean results are shown for rosiglitazone-treated cells from five different placentas vs their paired controls (SAM scatter plot in Figure 2, heat map in Figure 3 and Figure S1). Five independent EVCT cultures yielded similar results in four cases and a slightly different pattern for culture 1. A total of 139 genes (175 probe sets, 117 unique genes) were identified as having significantly different expression (p<0.05) in treated EVCTs. Overall, 114 genes (149 probe sets) were over-expressed (red) and 25 genes (26 probe sets) were under-expressed (green) in treated EVCTs. The complete gene list is presented in Table S1. Of the 20 genes possessing a putative PPARγ response element (Genomaxit Software GmbH, http://www.genomatix.de, accessed 2013), 17 are upregulated and 3 downregulated (Figure 4). All of them are reported to be expressed in the placenta, except for UPK1A, initially described as being involved in urothelial cell differentiation [32].

Functional network analyses

Expression profiles were analyzed with Ingenuity Pathway Analysis software. The top seven functional networks showing altered gene expression in rosiglitazone-treated EVCTs are shown in Figure 5. Functional areas included cell signaling, molecular transport and small-molecule biochemistry, cell growth, lipid metabolism, cell cycling, tissue morphology, skeletal and muscular system development and function, cancer, and cell death. The LOX gene was found to be part of networks 2, 5 and 7 (Figure 5).

The transcriptome data were confirmed for selected genes, namely those found here to be strongly regulated or known to be involved in placental development. RT-qPCR was applied to treated and paired control EVCT cultures distinct from those used for the microarray experiments (Figure S2). RNA levels of connexin 43 (CX43, GJA1), deleted in liver cancer 1 (DLC1), dipeptidyl peptidase 4 (DPP4), heme oxygenase 1 (HO-1, HMOX1), lysyl oxidase (LOX) and plasminogen activator inhibitor 1 (PAI-1, SERPINE1) following 24 h of exposure to rosiglitazone (1 μM) were between 1.7-fold (CX43) and 5.2-fold (DLC1) higher than in paired untreated cultures, while ADAM12 and PPARγ expression decreased by 0.2- and 0.4-fold, respectively. These differences were statistically significant for all the genes analyzed (LOX, ADAM12, p<0.05; DLC1 p≤0.01; CX43, DPP4, HO-1, PAI-1, PPARγ, p<0.001) and confirmed the GeneChip results (Figure 6A).

LOX expression in first-trimester EVCTs

Among the genes found to be upregulated in rosiglitazone-treated EVCTs, we further examined the role of LOXs. Both GeneChip and RT-qPCR experiments showed LOX to be upregulated in rosiglitazone-treated EVCTs, by 3.3-fold (p<0.05) and 2.6-fold (p<0.05), respectively (Table S1 and Figure 6).

Expression analysis of the LOX genes by RT-qPCR showed that rosiglitazone-induced PPARγ activation resulted in a significant increase in the following three of the five LOX isoforms: LOX (3.2-fold versus controls, p≤0.001), LOXL1 (1.6-fold, p<0.005) and LOXL2 (2.6-fold, p<0.005). No significant change in LOXL3 or LOXL4 RNA levels was observed (Figure 6B). We then focused on transcript and protein expression of the LOX, LOXL1 and LOXL2 genes in placental tissues and control EVCTs. The copy number of each isoform was determined by absolute qPCR in primary EVCT cultures. LOX and LOXL1 RNAs were present in equal quantities (about 800 copies/ng total RNA) (Figure 7A). In first-trimester placental villi and EVCTs, western blot revealed a band at about 30 kDa that might correspond to mature LOX, together with three bands at 34, 52 and 63 kDa that might correspond to mature, pro- and prepro-LOXL1, respectively (Figure 7B). LOX protein was weakly detected in villi and cultured EVCTs. LOXL1 was more abundant (2.3±0.5 fold increase, n = 3, p<0.01) in EVCTs than in villi, suggesting preferential expression of LOXL1 in EVCTs (Figure 7B). Pro-LOX and pre-LOXL1 were detected in conditioned medium (CM) and in the insoluble fraction of cell extracts (IF). The mature protein was detected in IF at 34 kDa. LOXL2 RNA was expressed in EVCTs (about 1700 copies/ng total RNA) but no significant signal was detected in cells or protein extracts with any of the antibodies tested. An immunofluorescence signal was obtained in term trophoblastic cells, used as a positive control (data not shown).

Immunolocalization of LOX isoforms in placental tissues and cultured cells

Immunohistochemical analyses of tissue sections from 8- to 9-WA villous columns showed a cytoplasmic and perinuclear pattern of LOX staining, while LOXL1 was mainly located within nuclei and nucleoli-like structures (Figure 8). Immunofluorescence data for cultured EVCTs were consistent with those obtained by immunohistochemistry. For LOX, immunocytochemistry showed stained spots suggesting the presence of granules throughout the cytoplasm (Figure 9A), whereas LOXL1 was mainly localized in the nucleus, with stronger labeling in the nucleolus (Figure 9B). We also observed pericellular LOX labeling. In rosiglitazone-treated cells we observed an increase in LOXL1 immunolabeling (Figure 9B), as well as cytoskeleton modifications, as shown by CK7 immunostaining (Figures 9A and 9B).
LOXs affect EVCT invasion

We then examined the effect of LOX inhibition on EVCT invasion. LOX activity was inhibited with β-aminopropionitrile (BAPN), a specific and irreversible inhibitor [33]. BAPN-treated EVCTs were nearly twice as invasive as control cells (100 μM, 174 ± 32%; 200 μM, 190 ± 9%; p<0.01; Figure 10A). No significant difference was observed between the effects of 100 and 200 μM BAPN. EVCT invasion was inhibited by 35% (p<0.01) following rosiglitazone treatment, and this effect was overcome by adding BAPN (Figure 10B). The ratios of BAPN-treated/control cells and rosiglitazone+BAPN/rosiglitazone-treated cells were similar, suggesting that the effect of BAPN remained at the same order of magnitude (1.6-fold increase in cell invasion) in the presence and absence of rosiglitazone.

Discussion

Cell invasion is a fundamental process during embryonic development [34], and also plays an essential role in tumorigenesis [35]. In humans, invasion of cytotrophoblasts into the maternal uterus is a temporally and spatially controlled process critical for successful placental and fetal development [3,36,37]. Defective trophoblast invasion has been implicated in complications of pregnancy such as intrauterine growth restriction and preeclampsia [7,9]. We have previously shown that activation of the nuclear receptor PPARγ by its specific synthetic agonist rosiglitazone and its natural ligands (15-deoxy-D-(12,14)-prostaglandinJ2 (15D-PGJ2), 15-hydroxy-eicosatetraenoic acid (15-HETE), 9- and 13-hydroxy-octadecenoic acid (HODE)) decreases human EVCT invasion in vitro [12,26,38,39]. Rosiglitazone belongs to the thiazolidinedione class of drugs, and is the first synthetic drug with high selectivity for the PPARγ isoform (Kd approximately 40 nM). In transfection models, rosiglitazone does not activate the other PPARγ isoforms (PPARα and PPARδ) at concentrations as high as 100 μM [20]. As we have previously reported, inhibition of human primary EVCT invasion by rosiglitazone is concentration-dependent, with 50% inhibition at 1 μM [12]. This concentration has been widely used to activate PPARγ in various cellular models, including...
adipocytes and trophoblasts [40,41]. In the 10-50 μM range, rosiglitazone has been reported to act through PPARγ-independent mechanisms [42,43,44].

Here, to identify differentially regulated genes that might play a role in placental trophoblast invasion, we used EVCTs isolated from 8–9 WA human placentas and compared gene expression patterns between these normally invasive EVCTs and rosiglitazone-treated EVCTs that display reduced invasiveness. Primary EVCTs were chosen because none of the available established human trophoblast cell lines display the same pattern of gene expression as primary cells [45]. The Achilles’ heel of this model is the dearth of available cells.

The pleiotropic function of PPARγ in the placenta [46] is reflected by the diversity of regulated genes in rosiglitazone-treated EVCTs (Table S1). This regulation might be direct or indirect. Among the 20 genes containing a putative PPARγ response element (Figure 4).

Figure 5. Microarray and transcriptome analyses of rosiglitazone-treated EVCTs compared to paired controls: top 7 networks. The 175 probe sets were loaded into Ingenuity Pathway Analysis software (IPA) and converted into gene networks. Genes shown in bold type are present in the input data list as upregulated (red) or downregulated (green). Genes involved in the network that are not included in the transcriptome results are shown in black. * genes with several probes present in the input data list.

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element (Figure 4), angiopoietin-like 4 (ANGPTL4), fatty acid binding protein 4 (FABP4) and plasminogen activator inhibitor 1 (PAI-1, SERPINE1) are direct PPARγ target genes [47,48,49].

Our findings highlight the differential regulation of several genes that might be involved in various aspects of placental development, including cell metabolism and invasive processes. The expression of numerous genes involved in fatty-acid metabolism and signaling was upregulated in rosiglitazone-treated EVCTs, including acyl-CoA synthetase long-chain family members 1 and 5 (ACSL1, ACSL5), elongation of very long chain fatty acid like 4 (ELOVL4), fatty acid-binding protein 4 and 5 (FABP4, FABP5), insulin induced gene 1 (INSIG1) and very low density lipoprotein receptor (VLDLR) or downregulated as monoglyceride lipase (MGLL). This supports PPARγ involvement in fatty acid metabolism in the human placenta, which is essential for normal fetal development [30,31].

Cell invasion is a highly integrated multistep process responding to extracellular stimuli and involving cell adhesion and motility. It requires the coordination of a wide spectrum of signaling molecules and regulation of cytoskeleton dynamics [52]. Invasive cells move into neighboring tissue in a process that involves extracellular matrix degradation and proteolysis [53]. Among candidate genes that might be involved in the EVCT invasion process and that were differentially expressed in rosiglitazone-treated cells were several that encode proteinases, namely a disintegrin and metalloproteinase (ADAM12), dipeptidylpeptidase 4 (DPP4) and pappalyisin 2 (PAPP2), PAI-1 (PAI-1, SERPINE1). ADAM12 has extracellular metallopeptidase and cell-binding functions and intracellular signaling properties [54]. Extracellular proteolysis could have a proinvasive effect by affecting insulin-like growth factor (IGF) signaling through cleavage of IGF-binding proteins [55,56] and epidermal growth factor (EGF) pathway via ectodomain shedding of membrane-tethered EGF-receptor ligands [57,58]. Interaction of ADAM12 with cell-surface integrins may induce actin cytoskeletal changes. ADAM12 may also mediate signals through its intracellular domain (for review see [54]). DPP4 is a marker of EVCTs with a non invasive phenotype, and its down-regulation is associated with migration or invasion [59,60]. Furthermore, DPP4 expression and activity are increased in preeclamptic placental tissues [61]. The metalloproteinase PAPPA2 cleaves IGFBP5 [62], potentially resulting in inhibition of EVCT invasion [63]. PAPPA2 is localized in the syncytiotrophoblast layer of placental villi and in invasive EVCTs [63,64]. It is overexpressed at the maternal-fetal interface in placental samples from women with preeclampsia [63]. Concerning PAI-1, induction of its expression by TNF-α is associated with reduced EVCT invasion and migration in the first-trimester placental explant culture model [65].

A number of cytokines, growth factors and their receptors have been shown to regulate human trophoblast invasiveness [66,67]. Here we observed differential expression of colony-stimulating factor 3 receptor (CSF3R), insulin-like growth factor 1 receptor (IGF1R) and interleukin 10 receptor (IL10RA). The granulocyte-colony-stimulating factor receptor (G-CSFR, CSF3R) is development-stage-specific and has been detected in EVCTs in situ [68]. IGF-1 has been shown to stimulate EVCT invasion through the α3β3 integrin signaling pathway [56]. IL10R is expressed by cytotrophoblasts at all gestational ages and its ligand (IL-10) has been reported to be an autocrine inhibitor of cytotrophoblast migration or invasion [59,60]. Furthermore, DPP4 expression and activity are increased in preeclamptic placental tissues [61]. The metalloproteinase PAPPA2 cleaves IGFBP5 [62], potentially resulting in inhibition of EVCT invasion [63]. PAPPA2 is localized in the syncytiotrophoblast layer of placental villi and in invasive EVCTs [63,64]. It is overexpressed at the maternal-fetal interface in placental samples from women with preeclampsia [63]. Concerning PAI-1, induction of its expression by TNF-α is associated with reduced EVCT invasion and migration in the first-trimester placental explant culture model [65].

Many different molecules and signaling pathways coordinate cell migration, but dynamic cytoskeleton reorganization is at the heart of this process [36,52]. This is illustrated here by the contraction of intermediate filaments in rosiglitazone-treated EVCTs (Figure 9). Rho family small guanosine triphosphate (GTP-binding proteins (GTPases) coordinate the cellular responses required for cell migration by regulating the actin cytoskeleton and affecting the organization of the microtubule and intermediate filament networks, as well as cell-substrate adhesion [70]. The Rho signaling pathway has been implicated in trophoblast motility (for review see [36]). The expression of several genes encoding members of this signaling pathway was found to be modulated by rosiglitazone, including Rho GTPase-activating protein (DLC1), Rho GTPase binding-protein (CDC42EP4) and Rho guanine nucleotide exchange factors (ARHGEF3, ARHGEF4 and NEF). DLC1 is essential for embryonic development. In mice, 

\[ \text{DLC1}^{-/-} \] embryos do not survive beyond E10.5. Defects in the neural tube, brain, heart and placenta are observed, as well as altered organization of actin filaments and focal adhesions in cultured 

\[ \text{DLC1}^{-/-} \] fibroblasts [71]. Expression of DLC-1 was
strongly upregulated in rosiglitazone-treated EVCTs. DLC1 is a potential tumor suppressor gene, as its expression reduces the migratory capacity of tumor cells [72]. In metastatic non-small-cell lung cancer cells (NSCLC), PPARγ overexpression has been shown to inhibit cell invasion and to be associated with DLC1 induction [73].

Gap junctional intercellular communication (GJIC) and connexin (Cx) expression are also involved in placental development [74,75]. Connexins are a family of integral membrane proteins that oligomerize into clusters of intercellular channels called gap junctions. Gap junctions allow direct intercellular communication and diffusion of ions and signaling molecules between contacting cells. The expression of both Cx43 (GJA1) and Cx40 (GJA5) was upregulated in rosiglitazone-treated EVCTs. Cx43 is involved in human trophoblast differentiation and cell fusion [76,77]. It has been detected in first-trimester villous trophoblasts and extravillous trophoblastic aggregated cells of the placental bed in situ [76,78], and also in cultured EVCTs in vitro [79]. It is interesting to note that Cx43 expression in breast cancer cells reduces their metastasis to lung [80]. In the human placenta, Cx40 is expressed in proliferative EVCTs of the cell column. Expression becomes weak in distal cell columns when trophoblasts migrate; Cx40 is then reexpressed in trophoblasts aggregated within the decidua [76,79].

According to Malassine and Cronier, Cx40 plays a critical role in the switch from a proliferative to an invasive phenotype by trophoblastic cells invading the endometrium [75].

Heme oxygenase-1 (HMOX-1) was also upregulated in rosiglitazone-treated EVCTs. This corroborates the report from Bilban et al. [81] describing HMOX-1 downregulation in human invasive EVCTs obtained from villous explant cultures by comparison with poorly invasive villous trophoblasts isolated from first-trimester placentas. Moreover, the latter authors identified HMOX-1 as a negative regulator of trophoblast motility, acting via upregulation of PPARγ protein levels and activity in choriocarcinoma cell lines.

A number of trophoblastic genes were also downregulated following rosiglitazone treatment (Table S1) including PPARγ itself as reported in murine trophoblast stem cells [41], suggesting the existence of a negative feedback loop.

Among the known PPARγ targets in the mouse trophoblast is the mucin gene MUC1 [40]. In humans, MUC1 is overexpressed in severe preeclampsia, and its overexpression suppresses trophoblast cell invasion [82]. Here, we found that MUC1 expression was very weak in the rosiglitazone-treated EVCTs. This corroborates the report from Bilban et al. showing that MUC1 mRNA and protein levels increase during human placental development and are barely detectable in first-trimester placentas [83].

Among the most strongly upregulated genes, we selected LOX for further investigation on the basis of published data, notably because its expression and activity are dependent on oxygen levels, and because it is known to have a role in tumor suppression, cell migration and invasion [30,84,85,86,87]. These properties indicate that LOX might play a role in trophoblast invasion during the first trimester of pregnancy when oxygen tension increases. Our analysis confirms earlier reports of the presence of LOX in human placenta in situ [88] and of LOXL1 transcripts in EVCTs in vitro [81]. We also detected LOXL1 transcripts in EVCT primary cultures, although protein expression was very low as reported by others at this stage of pregnancy [88]. In vitro, LOX and LOXL1 proteins were detected in EVCT primary cultures from 8- to 9-WA human chorionic villi. Confocal microscopy results further suggest that LOX protein could be concentrated in the cytoplasm, particularly in granules, while LOXL1 would be located mainly in the nuclei and nucleoli, probably after secretion, processing and internalization, as described by Hayashi K, et al. [89]. These observations are in keeping with the lower intracellular protein levels of LOX compared to LOXL1 in villi and EVCTs, as shown here by western blotting. In culture media, LOX and LOXL1 were detected as pro-proteins (about 50 kDa). The absence of the mature enzymes, produced by bone morphogenic protein (BMP) cleavage in the extracellular matrix [90], could be explained, in the case of LOXL1, by cellular uptake. The presence of LOX in the cell surroundings (immunocytochemistry) and in the insoluble fraction (western blot) suggests it may be trapped in the extracellular matrix secreted by EVCTs. The nuclear localization of LOX suggests that it may have a role in the regulation of gene expression. Binding of LOX to histones H1 and H2 has been observed in vivo and in vitro, and has been suggested to modify the degree of chromatin compaction [91,92]. The differential protein expression and subcellular localization of the LOX isoforms
suggest that they could act through different mechanisms in EVCTs.

Inhibition of LOX enzymatic activity by BAPN enhanced the invasiveness of primary cultured EVCTs, while LOX upregulation by rosiglitazone was associated with a decrease in EVCT invasiveness. Furthermore, inhibition of EVCT invasiveness by rosiglitazone was totally overcome by BAPN treatment. Together, these findings suggest that the activity of LOX and/or LOXL1 negatively regulates cytotrophoblast invasion. LOX activity might reduce invasiveness through its ability to increase matrix stability.

LOX expression has been linked to both tumor progression and tumor suppression [93,94]. In tumor cells, LOX activity promotes cell invasion and migration [30,84,86]. In contrast to active LOX, the LOX propeptide (LOX-PP) generated during the course of BMP-1-mediated LOX activation in the ECM acts as a tumor suppressor through multiple signaling pathways [85,87].

Together, these observations suggest that LOXs regulate cell invasiveness through different mechanisms in normal and tumor cells. Thus, the available human invasive trophoblast cell lines, which consist of choriocarcinoma cells and transformed trophoblasts, are unlikely to mimic normal trophoblastic cell behavior, as pointed out by Bilban et al. [45].

The effects of global inhibition of LOX family activity by BAPN strongly suggest a role of LOXs in trophoblast invasion. This could be confirmed by selective inhibition of each LOX isoform with a siRNA strategy, such experiments remain to be performed.

In conclusion, our study provides the first transcriptome of PPARγ target genes in first-trimester primary cultures of human invasive EVCT, and supplies further evidence that trophoblast invasiveness is controlled by the PPARγ pathway, via novel downstream target genes. Among the latter, we show that LOX and/or LOXL1 activity may be involved in negative regulation of human trophoblast invasion.

Materials and Methods

Ethics statement

The study conformed to the Declaration of Helsinki. The placentas used for this study were obtained with the patients’ written informed consent, and the protocol was approved by our local ethics committee (CCPRB Paris Cochin n° 18-05). Placental tissues were obtained from women undergoing legal and voluntary termination of a normal pregnancy during the 8-9th week of amenorrhea (WA), at Broussais hospital (Paris, France).

EVCT isolation, purification and treatment

EVCTs were isolated from first-trimester chorial villi (n = 6) as previously described [10,11], with modifications. Cells were plated in culture dishes (Techno Plastic Products, Switzerland) coated with Matrigel® (7 μg/cm²; Collaborative Biomedical Products, Le Pont de Claix, France) at 5×10⁴ cells/cm² in DMEM-F12 culture medium containing Glutamax, 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Illkirch, France). The yield was up to 3.5×10⁵ non-proliferative EVCTs per gram of chorial villi (up to 5 g at 8–9 WA). After 2 h at 37°C in a humidified incubator with 5% CO₂, non-adherent cells were removed by washing. For cultures used in microarray experiments, RT-qPCR, immunocytochemistry and immunoblotting, the medium was replaced after a further 24 h and 1 μM rosiglitazone (Cayman, Ann Arbor, MI) dissolved at 1 mM in ethanol was added. There was no effect of 0.1% ethanol (vehicle) on EVCT cultures (data not shown). Rosiglitazone-treated and control cells were trypsinized 24 h later for RNA assays or 48 h later for protein assays.

Figure 8. Immunohistological localization of LOX and LOXL1. Experiments were carried out on PFA-fixed sections of 8-WA placental villi, using specific antibodies; non-specific rabbit IgG was used as a control. Immunostaining was performed with an universal streptavidin-peroxidase kit (Dako). doi:10.1371/journal.pone.0079413.g008
Microarray analysis
EVCT primary cultures from first-trimester placentas (rosiglitazone-treated and matched vehicle controls, n = 5 placentas) were obtained as described above. RNA was extracted using the TRIzol® reagent (Invitrogen) as described for RT-PCR, and then purified using the RNeasy® Mini kit (Qiagen S.A., Courtaboef, France). RNA integrity and purity were checked using a 2100 Bioanalyzer with the RNA 6000 LabChip kit (Agilent Technologies, Massy, France). The GeneChip (U133A 2.0, Affymetrix, Inc., Santa Clara, CA), which analyzes 14 500 genes with 22 000 probe sets, was used according to the manufacturer’s instructions. Data were processed with the Expression Analysis algorithm of the Affymetrix Microarray suite (version 4.0). CEL files were imported into BRB Array Tools software (http://linus.nci.nih.gov/BRB-ArrayTools.html) for normalization with the RNA algorithm and for further analysis. Differences in gene expression between rosiglitazone-treated and vehicle control EVCTs were analyzed by using significance analysis of microarrays (SAM) [95] after applying a filtering threshold: i) less than 20% of expression data have at least 1.5-fold change in either direction from gene’s median value; ii) percent of data missing or filtered out exceeds 50% which selected 3354 probes. Statistical comparison was used to identify over- and under-expressed genes, focusing on genes that exhibited at least a 1.5-fold change, with a false discovery rate (FDR) of 3%.

Functional analysis
Ingenuity Pathway Analysis software (IPA version 9.0, http://www.ingenuity.com/ accessed 2011) was used to compute the statistical differences in gene sets between rosiglitazone treated and vehicle control EVCTs and to define cellular function and metabolic pathway involvement.

Relative quantitative RT-qPCR (RT-qPCR)
Relative RT-qPCR was used to compare the expression of selected genes in a new set of 6 independent experiments. Total RNA was extracted with the TRIzol® reagent, according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized from total RNA (0.5 µg) using the Superscript II Reverse Transcriptase kit (Invitrogen). All RT-qPCR reactions were performed in an ABI Prism 7300 Sequence Detection system (Applied Biosystems, Courtaboef, France) using a cDNA dilution corresponding to 6.25 ng of RNA with the SYBR® Green PCR kit (Applied Biosystems). Each experiment was performed in duplicate for each gene. An initial denaturation step at 95°C for 10 min was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and by a three-step dissociation phase of 15 s at 95°C, 30 s at 60°C and 15 s at 95°C to eliminate possible artifacts such as oligonucleotide dimers. The primers used (Eurogentec, Angers, France) are described in Figure S2. Cytokeratin 7 (CK7), a trophoblast-specific intermediate filament in human placenta, and 18S RNA were used as endogenous RNA controls and gave similar results. Results normalized to 18S RNA are expressed as a percentage of the calibrated control (untreated sample).

Absolute quantitative RT-qPCR
Absolute RT-qPCR was used to estimate the LOX isoform RNA copy number in 48-h primary EVCT cultures, using specific primers (Figure S2B); cDNAs of LOX and LOXL1 were obtained by reverse transcription of total RNA extracted from placental villi and amplification by Go Taq DNA polymerase (Promega, Madison, WI). The specificity of each DNA was determined by sequencing (Cogenics, Meylan, France). Amplified cDNA from
three RT-PCR tubes for each isoform was purified using Nucleospin (Macherey-Nagel, Düren, Germany) and the DNA concentration was measured with a Nanodrop® ND-1000 spectrophotometer. Copy numbers were determined by using the Avogadro number. Standard curves (10^2 to 10^6 cDNA copies) were prepared for each isoform in parallel with EVCT cDNA samples (n = 5), using the SYBR® Green PCR kit as described above (primers in Figure S2A).

Immunoblotting
EVCTs were trypsinized after 72 h of culture, harvested, washed in PBS and frozen at -80°C. Proteins were extracted from EVCTs pellets with the M-PER protein extraction reagent (Pierce) according to the manufacturer’s instructions (http://www.piercenet.com/instructions/2160805.pdf). The M-PER pellet, representing the insoluble fraction (IF), was taken up in Laemmli buffer and sonicated [96]. Cell conditioned medium (CM) was collected from two dishes, centrifuged and then concentrated in a 10-kDa Ultra filter unit (Amicon Ultra, Millipore) by centrifuging at 3000 g for 20 min. Thirty and forty micrograms of protein from cell pellets and conditioned medium, respectively, and the whole insoluble fraction, were analyzed by western blot as previously described [97]. Twenty microliters of extracted MatrigelTM protein was run as a control. The PolyScience polycrylindene fluoride membrane (PerkinElmer, Courtaboeuf, France) was blocked in Tris-buffered saline, 0.1% Tween 20 (TBS-T) then incubated overnight at 4°C with specific rabbit polyclonal antibodies against LOX, LOXL1 or LOXL2 (2.5 mg/mL) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. The specific bands were revealed by chemiluminescence (Western Blotting Chemiluminescent; Pierce) at room temperature then visualized by autoradiography (Kodak Biomax MR film). After three TBS-T washes, the membrane was incubated with a hors eradish peroxydase-conjugated goat anti-rabbit IgG (0.03 mg/mL; Jackson ImmunoResearch Laboratories) at room temperature, followed by two PBS washes. The slides were mounted with Vectashield® (Vector Laboratories, Burlingame, CA) and examined with a Leica confocal microscope (IFR71-IMTCE Imaging Facility, Paris Descartes University).

Invasion assay
EVCTs (25×10^4 cells), isolated as described above, were plated in the upper chamber of 8-micron Transwell inserts (6.5 mm diameter; BD Falcon, le Pont de Claix, France) coated with 60 μg MatrigelTM and incubated for 2 hours in EVCT culture medium. The cultures were then washed and treated for 48 h with complete medium containing 100 or 200 μM BAPN (Sigma), a specific LOX inhibitor [33]; BAPN stock solution was 100 mM in water. Medium without BAPN was used as a control. The cells were washed and fixed with 4% paraformaldehyde for 20 min at room temperature then visualized by immunostaining with anti-CK7 as described above. The filters were cut out from the inserts and placed on a glass slide. Nuclei were labeled with fluorescent 4’,6-diamino-2-phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA) and examined with a Leica confocal microscope (IFR71-IMTCE Imaging Facility, Paris Descartes University).

Statistical analysis
Values reported here are the mean and SEM of 4–6 primary cultures, each derived from a different placenta. ANOVA and the Mann-Whitney test were used to compare treated and control EVCT cultures for mRNA expression, the number of LOX isoform RNA copies and invasive capacity. Differences were considered significant when p<0.05 (ANOVA).

Supporting Information
Figure S1 Heatmap of the 175 probe sets (139 genes, 117 unique genes) selected with the SAM procedure, together with the probes and gene names.

(TIF)

Figure S2 Forward and reverse oligonucleotides used for absolute and relative qPCR.

(TIF)
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Table S1 The complete list of upregulated and downregulated genes in rosiglitazone-treated EVTcs. (DOC)

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**Author Contributions**

Conceived and designed the experiments: NS DEB TF. Performed the experiments: NS SB EC. Analyzed the data: NS SD JB PD TF. Contributed reagents/materials/analysis tools: SD CR BS PD KSKF KC. Wrote the paper: NS JB TF.

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