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Short Communication

Notch1 and the activated NOTCH1 intracellular domain are expressed in differentiated trophoblast cells

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Abstract

The Notch signalling pathway regulates proliferation, cell death and cell type specification that is critical for organogenesis. Mouse models carrying mutations in the Notch signalling pathway display defects in development of the placenta, suggesting that this pathway is required for placental development. In particular, *Notch1* mutant embryos exhibit abnormal placental morphogenesis and arrest early in development. However, expression of *Notch1* gene has not been detected during placental development. Trophoblast stem cells are derived from the precursor of the placenta and express *Notch1*. We report that *Notch1* is also expressed in differentiated trophoblast cells. Under standard differentiation conditions, *Notch1* expression ceases by day 6. Furthermore, the activated NOTCH1 intracellular domain is enriched at the nucleolus of trophoblast stem cells and differentiated trophoblast cells. Our results suggest that NOTCH1 is active in both trophoblast stem cells and differentiated trophoblast cells.

Keywords: differentiation; FGF4; Notch1; placenta; trophoblast stem cell

1. Introduction

During development, the Notch signalling pathway plays a critical role in patterning tissues by regulating proliferation, cell death and specifying cell fate determination (Bray, 2006; Kopan and Ilagan, 2009). The Notch receptors and ligands are transmembrane proteins involved in local cell–cell signalling (Kopan and Ilagan, 2009). Interaction between the receptor and ligand results in cleavage of the intracellular domain of NOTCH and release of an activated NOTCH intracellular domain (Gasperowicz and Otto, 2008). In the nucleus, the activated NOTCH intracellular domain binds to the promoter of NOTCH target genes via interaction with the transcription factor, RBPjk (recombination signal-binding protein 1 for J-kappa). Four Notch receptors (*Notch1–Notch4*) and five Notch ligands (*Dll1*, *Dll3*, *Dll4*, *Jag1* and *Jag2*) have been identified in mice. Loss-of-function mutations in receptors, *Notch1*, *Notch2*; ligands, *Jag1*, *Dll4*; transcription factor, *Rbpjk* or downstream targets *Hey1*/*Hey2*, and *Mash2*, result in embryonic lethality at midgestation, suggesting that the pathway is required for normal embryogenesis (Guillemot et al., 1995; Oka et al., 1995; Xue et al., 1999; Krebs et al., 2000; Fischer et al., 2004; Gale et al., 2004).

Embryonic arrest at midgestation is often associated with placental insufficiency, and placental abnormalities have been reported in *Notch* mutant embryos (Conway et al., 2003; Gasperowicz and Otto, 2008). In *Notch2* mutant embryos, the maternal sinuses fail to expand properly, thus limiting the efficiency of exchange between the maternal and fetal interface (Hamada et al., 2007). In *Notch1* homozygous mutant embryos, embryonic blood vessels fail to invade the chorion, resulting in failure to form a functional placenta (Krebs et al., 2000). However, expression analysis of *Notch* genes in the developing placenta revealed expression of only *Notch2* in two of the differentiated trophoblast cell types of the placenta, spongiotrophoblast and trophoblast giant cells, by *in situ* hybridization (Nakayama et al., 1997). In human placenta, NOTCH1 is expressed in syncytiotrophoblast and cytotrophoblast cells (De Falco et al., 2007). Furthermore, expression level of NOTCH1 protein is decreased in placentas from pre-eclampsia-associated pregnancies, suggesting that normal NOTCH1 expression is required for normal human placental function (Cobellis et al., 2007). Thus, although Notch1/NOTCH1's expression has not been reported in the mouse placenta or in differentiated trophoblast cells in mice, the mouse *Notch1* mutant phenotype and human placental studies suggest a conserved requirement for NOTCH1 in placental development.

Trophoblast stem cells are derived from the precursor of the placenta and can be induced to differentiate into the trophoblast cell types of the mature placenta. Components of the Notch signalling pathway, including *Notch1* and *Notch2*, were detected in trophoblast stem cells by RT (reverse-transcription)-PCR (Cormier et al., 2004). However, it is not clear if *Notch1* expression is maintained during induced differentiation of trophoblast stem cells or if the NOTCH1 receptor is activated in these cells. To determine if *Notch1* is present during trophoblast differentiation, we examined expression of *Notch1* mRNA and NOTCH1 receptor activation in trophoblast stem cells and differentiating trophoblast cells. We show herein that *Notch1* is expressed in trophoblast stem cells and differentiating trophoblast cells. Furthermore, we report that the activated NOTCH1 intracellular domain is expressed in trophoblast stem cells and in differentiated trophoblast cells, indicating activation of the Notch pathway during trophoblast differentiation.

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Abbreviations: Jag, Jagged; Dll, delta-like; FGF4, fibroblast growth factor 4; NICD, Notch intracellular domain; RBPjk, recombination signal-binding protein 1 for J-kappa; RT-PCR, reverse-transcription PCR.

2. Materials and methods

2.1. Trophoblast stem cell culture

Trophoblast stem cells (courtesy of J. Rossant) were cultured as described by Tanaka (2006). All differentiation experiments were conducted on gelatin-coated plates, in the absence of a feeder layer.

Trophoblast cells were classified by their morphology: Trophoblast stem cells grew in tight epithelial colonies and had a large nucleus/cytoplasm ratio. Differentiated trophoblast stem cells, which we presumed includes spongiotrophoblast cells, appeared as mononuclear cells with a larger cytoplasmic area. Syncytiotrophoblast cells had two to four nuclei in a single cytoplasm, and the giant cells were large cells with large nuclei and large cytoplasm in comparison with other cells in the culture.

2.2. RT-PCR analyses

Trophoblast stem cells cultured 0, 2, 4 and 6 days after FGF4 (fibroblast growth factor 4) and heparin withdrawal were trypsinized, pelleted and suspended in TRIZOL (Invitrogen). The RNA was extracted according to the product's protocol. The RNA was treated with DNase (Invitrogen) according to the company's manual. cDNA was synthesized using Superscript III (Invitrogen).

RT-PCR analysis was conducted on cDNA using the following primer sets: *Cdx2* TCTCCGAGAGGCAGGTTAAA/GCAAGGAGGTCACAGGACTC, *Mash2* TGCGCTCCGCGGTAGAGTAC/TGCTTTCCTCCGACGAGTAGG, *Gcm1* ACGAAGAGATGGCATGCATG/CTTGTGACATTACACCTGGC, *Pl1* GCTGCATTAAAAGGGACATC/CCATTATAGCTCTACATAACTGAGG, *Pl2* TGCTGTCAAGAACAAAGGAG/ATTTGGGGGTAAGATGACAA, *Notch1* TGCCACTATGGTTCCTGTAA/GGTTACTGTTGCACTCGTTG, *Rbpjk* ATTACGGGCAGACTGTCAAG/AAGTTCAAGCATTGCTACGTC, *Beta-actin* AGCTTCTTTGCAGCTCCTT/CACCCACATAGGAGTCCTTC. All PCRs were conducted using the following conditions: 94°C for 3 min followed by 34 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 30s.

Three independent differentiation experiments were conducted, and three independent RNA samples were collected at each time point. At least two cDNA synthesis reactions were performed for each RNA sample, and at least three PCR reactions were performed for each gene. A gene was determined to be expressed in an experiment when a band of the expected size was detected in two or more PCR reactions.

2.3. Immunocytochemistry

Cells plated on four-well slide (Nunc) were fixed in 4% formaldehyde for 15 min and blocked in 5% normal serum in PBS with 0.3% Triton X-100 (Fisher Scientific) for 60 min. Samples were then treated with rabbit anti-NICD1 (Abcam ab8925, 1:300), in PBS/Triton for either 60 min at room temperature or overnight at 4°C. For fluorescence, Alexa fluora secondary antibody (Invitrogen, 1:500) was used for 60 min at room temperature in the dark. Samples were mounted in mounting medium with DAPI staining (Vector Labs).

3. Results and discussion

3.1. Notch1 and Rbpjk are expressed in differentiating trophoblast stem cells

In vitro trophoblast stem cell expansion and maintenance is dependent on the addition of FGF4 into the culture medium. In the absence of FGF4, trophoblast stem cells differentiate into morphologically and molecularly distinct differentiated trophoblast cells (Tanaka et al., 1998). We performed RT-PCR analysis to analyse expression of *Notch1* in trophoblast stem cells and differentiated trophoblast cells 2, 4 and 6 days after FGF4 withdrawal. Trophoblast stem cells express the transcription factor, *Cdx2*, which is rapidly down-regulated after FGF4 withdrawal (Murohashi et al., 2010). We found expression of *Notch1*, *Rbpjk* and the trophoblast stem cell marker *Cdx2* in the presence of FGF4 (Figure 1A), as reported by Hughes et al. (2004) and Cormier et al. (2004). Similar to what has previously been reported (Hughes et al., 2004), we found a few differentiated cell

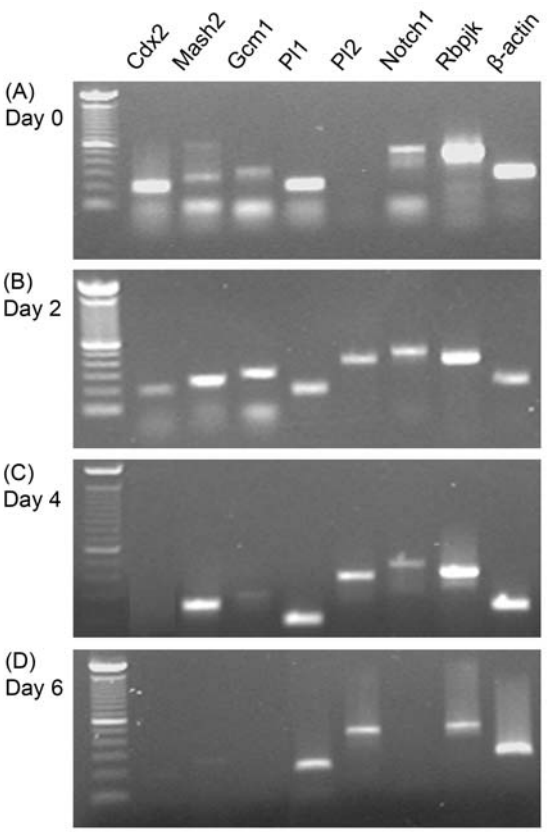

Figure 1 ***Notch1*** **and** ***Rbpjk*** **are expressed in trophoblast stem cells and differentiated trophoblast cells**

Representative RT-PCR analysis showing expression of *Cdx2*, *Mash2*, *Gcm1*, *Pl1*, *Pl2*, *Notch1* and *Rbpjk* in trophoblast stem cells cultured in the presence of FGF4 (**A**) or in the absence of FGF4 after 2 (**B**), 4 (**C**) and 6 (**D**) days. A 100-bp marker and the β-actin-positive control are also shown. All PCR analyses were repeated three to five times to confirm expression, and three different differentiation experiments were conducted to confirm the expression pattern observed.

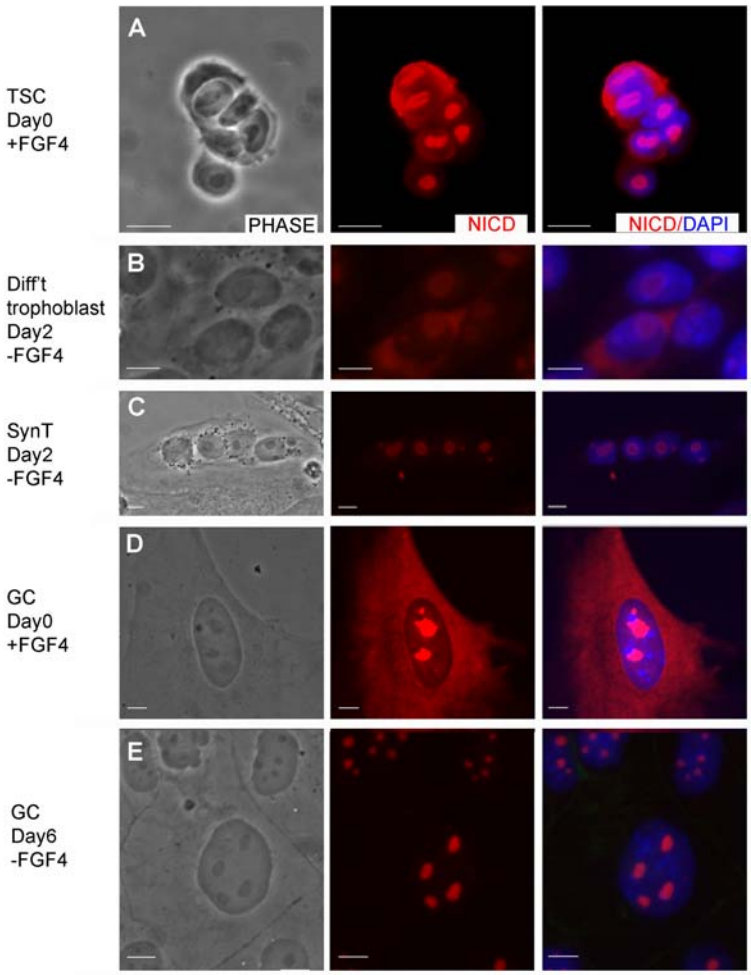

Figure 2 **The activated NOTCH1 intracellular domain is expressed in trophoblast stem cells and differentiated trophoblast cells**
Representative images of TSC (trophoblast stem cells) (**A**, **D**) and differentiated trophoblast cells (**B**, **C**, **E**) after immunocytochemistry with an antibody to reveal expression of the activated NOTCH1 intracellular domain. Expression of the activated NOTCH1 intracellular domain (red) was found in the cytoplasm and nucleus of trophoblast cells cultured in the presence of FGF4 (**A** and **D**) or in the absence of FGF4 for 2 (**B** and **C**) and 6 (**E**) days. The nucleus was stained with DAPI (blue). (**A**–**E**) The strongly stained nuclear foci correspond to the dense nucleolar body in the phase-contrast images. Diff't, differentiated; SynT, syncytiotrophoblast cells; GC, giant cells. Scale bar=10 μm.

types in our cultures under optimal trophoblast stem cell culture conditions (Figure 2D). We also occasionally detected expression of differentiated cell markers such as *Mash2*, a spongiotrophoblast cell marker, *Gcm1*, a syncytiotrophoblast cell marker and the giant cell marker *Pl1* in the presence of FGF4 (Figure 1A).

During trophoblast stem cell differentiation, we found reduced (day 2; Figure 1B) and absent (days 4 and 6; Figures 1C–1D) expression of *Cdx2* after FGF4 withdrawal. Concomitantly, we found that expression of the spongiotrophoblast marker, *Mash2*, and the syncytiotrophoblast marker, *Gcm1*, increased after 2 days of FGF4 withdrawal and were absent by 6 days of FGF4 withdrawal (Figure 1D). In contrast, *mRNA* for the giant cell markers *Pl1* and *Pl2* increased after 2 days of FGF4 withdrawal and was maintained through 6 days after FGF4 withdrawal (Figures 1B–1D). Consistent with the findings of others, *Mash2* and *Gcm1* expression was tightly regulated during trophoblast differentiation (Hughes et al., 2004). However, we detected expression of the giant cell marker *Pl1* earlier in other studies. This could be due to differences in our culture conditions or in the sensitivity of our assay.

Nonetheless, we found that the expression profile of *Notch1* after FGF4 withdrawal was similar to that of *Mash2* and *Gcm1*. Thus, *Notch1* expression was detected 2 and 4 days after FGF4 withdrawal and absent at day 6 (Figures 1B–1C). Under the same culture conditions, the transcription factor *Rpbjk* was detected throughout differentiation, similar to *Pl1* (Figures 1A–1D). Our data suggest that *Notch1* is expressed in trophoblast stem cells and in differentiating trophoblast cells. Of the differentiated trophoblast cell types, *Notch1* expression corresponded with markers of spongiotrophoblast and syncytiotrophoblast cells. The fact that *Notch1* mRNA cannot be detected by *in situ* hybridization *in vivo* (Nakayama et al., 1997) suggests that this gene may be expressed in a few cells at the stages analysed or that the methods used were not sensitive enough.

3.2. Activated NOTCH1 intracellular domain localizes to the nucleolus of trophoblast stem cells and differentiated trophoblast cells

To determine if NOTCH1 protein is present and activated in trophoblast stem cells and/or differentiated trophoblast cells, we examined expression of the activated NOTCH1 intracellular domain in trophoblast stem cells and in trophoblast cells cultured 2, 4 and 6 days after FGF4 withdrawal. Immunocytochemistry with an anti-activated NOTCH1 intracellular domain antibody revealed expression of activated NOTCH1 intracellular domain in the cytoplasm and nucleus of trophoblast stem cells (Figure 2A) and differentiated trophoblast cells (Figures 2B–2E). The nuclear signal was associated with punctated foci, which corresponded to the nucleolus, a dense body that is visible by phase contrast microscopy (Figures 2A–2C). In the few differentiated giant cells found in the presence of FGF4, we detected activated NOTCH1 intracellular domain in the cytoplasm and the nucleus (Figure 2D). However, in differentiated trophoblast giant cells found 6 days after FGF4 withdrawal, expression of activated NOTCH1 intracellular domain was only detected in the nucleus (Figure 2E). Our results suggest that the Notch signalling pathway is activated in both trophoblast stem cells and differentiated trophoblast cells.

Intriguingly after 6 days of FGF4 withdrawal, we did not detect *Notch1* mRNA by RT-PCR, yet found nucleolar accumulation of the activated NOTCH1 intracellular domain. Nucleolar accumulation of activated NOTCH1 may be required for interaction with proteins that have previously been shown to regulate protein degradation in the nucleolus (Welcker et al., 2004).

4. Conclusion

We have shown that *Notch1* is expressed in trophoblast stem cells and in differentiated trophoblast cells. We further show that NOTCH1 is activated in trophoblast stem cells and differentiated cells. Our data, together with those of Krebs et al. (2000), suggest that the NOTCH1 receptor is active during development of the placenta. Future studies with reporter strains such as the Notch pathway activity sensor (Souilhol et al., 2006) may shed insight into the location of cells with NOTCH activity during *in vivo* placental development.

Author contribution

Loydie Anne Jerome-Majewska conceived of the project and coordinated the experiments. Didem Sarikaya performed and analysed the experiments. Both authors contributed to writing the manuscript.

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