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NOSTRIN: A novel modulator of trophoblast giant cell differentiation

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ABSTRACT

Differentiation-dependent expression of NOSTRIN in murine trophoblast cells prompted investigation on NOSTRIN's function in trophoblast differentiation. We show here that NOSTRIN levels increased in both mouse and rat placenta during gestation. NOSTRIN expression was not co-related to expression of eNOS precluding its eNOS mediated function. NOSTRIN transcripts were identified in trophoblast cells of the placenta, predominantly in trophoblast giant cells (TGC). Precocious over-expression of NOSTRIN during differentiation of trophoblast stem cells led to up-regulation of genetic markers associated with invasion (*Prl4a1, Prl2a1*) and TGC formation (*Prl2c2*, *Prl3d1*, *Prl3b1*). The functional consequence of NOSTRIN over-expression was increased TGC formation and trophoblast cell invasion. Furthermore, number of polyploid TGCs that arise by endoreduplication, were higher in presence of NOSTRIN. Early induction of NOSTRIN was associated with substantial decrease in G/F actin ratio and augmentation of N-WASP-Dynamin-NOSTRIN ternary complex formation that might be partially responsible for nucleation of actin filaments. NOSTRIN also formed a complex with Cdk1 and increased phosphorylation of T14 and Y15 residues that inhibits cytokinesis. Interestingly, SH3 domain deleted NOSTRIN was ineffective in eliciting NOSTRIN's function in differentiating trophoblast cells. These findings demonstrate that NOSTRIN potentiates trophoblast differentiation towards TGC trajectory that is critical for hemochorial placentation.

1. Introduction

Development of the placenta is initiated with the differentiation of the trophectoderm cells that forms the outer layer of the blastocyst (Rossant and Cross, 2001). Following attachment of the blastocyst to uterine epithelium the trophectoderm cells give rise to a niche of self-renewing multipotent progenitor cells, called trophoblast stem cells, which upon differentiation forms specialized trophoblast sublineages leading to formation of a functional placenta. Each branch of the trophoblast lineage develops specialized functions required for successful pregnancy (Simmons and Cross, 2005). Disruptions in trophoblast development can lead to intrauterine growth retardation, early pregnancy loss or various pregnancy related disorders (Silva and Serakides, 2016). These represent serious health problems whose etiologies are not sufficiently understood. A better understanding of trophoblast differentiation is therefore critical and will pave the way to understand developmental defects and pregnancy complications arising from inadequate placentation. Derivation of murine trophoblast stem cells (Tanaka et al., 1998) has provided an excellent model for dissecting the molecular mechanisms governing cell fate decisions and trophoblast differentiation *ex vivo*.

Differentiation of various trophoblast lineages during development of the mouse placenta is elegantly reviewed by Hemberger and Cross (2001), Baines and Renaud (2017). In mice, two placental regions are evident, a) junctional zone and b) labyrinth zone. Following implantation mural trophectoderm gives rise to primary TGC that encapsulate the entire conceptus except for the polar trophectoderm. Primary TGCs are the first definitive trophoblast cells to differentiate and arise by continuous endoreduplication of DNA despite cessation of cell division. Primary TGCs disappear as gestation progresses. The polar trophectoderm differentiates and forms a cylindrical structure containing two separate regions: ectoplacental cone and the extra-embryonic ectoderm. Trophoblast stem cells (TSC) within the ectoplacental cone differentiate into the distinct regions of the junctional zone. Secondary TGCs are differentiated from TSCs located at the periphery of the ectoplacental cone and line the boundary of the decidua and

Abbreviations: ICM, Inner cell mass; TE, Trophoectoderm; TSC, Trophoblast stem cell (TSC); NOSTRIN, Nitric Oxide Synthase Trafficking INducer; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PE, pre-eclampsia; PIH, pregnancy-induced hypertension; JZ, Junctional zone; LZ, Labyrinth zone; TGC, trophoblast giant cell; MEF, mouse embryonic fibroblasts

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placenta. TSCs also differentiate into spongiotrophoblast cells that form a sandwiched layer between the secondary trophoblast giant cells and the labyrinth zone. Glycogen cells of the junctional zone appear during the last half of pregnancy and are said to be the source of invasive trophoblast cells. TSCs located within the extra-embryonic ectoderm differentiate following the chorio-allantoic fusion to form the labyrinthine trophoblast cells (Rossant and Cross, 2002).

In the course of exploring novel molecular regulators of trophoblast differentiation, we investigated the potential role of NOSTRIN (Nitric Oxide Synthase Trafficking INducer) in trophoblast cells. NOSTRIN is classically known as an endothelial cell protein that possesses both eNOS dependent and independent functions (Zimmermann et al., 2002; Icking et al., 2005; Schilling et al., 2006; Choi et al., 2005; Chakraborty and Ain, 2017). Being an adaptor protein NOSTRIN interacts with various other proteins and depending on cellular context it exhibits its various functional cues (Kovacevic et al., 2015; Kovacevic et al., 2012; Chakraborty and Ain, 2017). Data from our lab (Chakraborty and Ain, 2017) demonstrated that NOSTRIN over-expression in endothelial cells can lead to a reduction in NO levels and also affected eNOS activation by phosphorylation. Endothelium derived relaxing factor, NO is considered as a primary mediator for lowering of vascular resistance during hemodynamic changes associated with pregnancy (Anumba et al., 1999). In agreement with the demonstrated function of NOSTRIN, it was found to be significantly up-regulated in placentas and umbilical vessels of women with pre-eclampsia (PE) and pregnancy-induced hypertension (PIH) with a reduction in NO production and eNOS activity (Xiang et al., 2009, 2006, 2005). Our previous data on NOSTRIN mediated effects on endothelial cell-secreted proinflammatory cytokines and increase in NOSTRIN expression in PIH/PE led us hypothesize for a potential role of NOSTRIN in placental development. While investigating the cellular source of NOSTRIN in placenta, we found predominant expression of NOSTRIN transcripts in TGCs located in the junctional zone of the placenta. These results prompted our investigation on NOSTRIN's function in trophoblast cells using trophoblast stem cell differentiation as a model.

Our finding identifies NOSTRIN as a modulator of placental trophoblast development. We demonstrate here that NOSTRIN is a significant novel contributor promoting TSC differentiation towards the TGC pathway, a function that requires SH3 domain of NOSTRIN.

2. Methods

2.1. Animals and tissue collection

To obtain mouse and rat placental tissues, sexually matured female Swiss albino mice or Sprague-Dawley rats obtained from Indian Institute of Chemical Biology (IICB) animal house were caged overnight with fertile males. Day 0.5 of pregnancy was designated by the presence of either copulatory plug in the vagina for mice or spermatozoa in morning vaginal smears in rats. Utero-placental tissues were dissected out from timed pregnant females on different days of gestation and either snap frozen in liquid nitrogen for RNA isolation or frozen in dry-ice chilled heptane for *in situ* hybridization. All tissue samples were stored at −80 °C until use. IICB Animal Care and Ethics Committee approved all procedures for handling and experimentation with animals as per guidelines set forward by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (http://cpcsea.nic.in).

2.2. Cell culture, transfection and differentiation

Mouse TSCs were kind gift from Prof. Janet Rossant, Hospital for Sick Children, Toronto, Canada. TSCs were cultured in 70% mouse embryonic fibroblast conditioned medium and 30% media, containing RPMI-1640 (Sigma Aldrich, USA) 20% FBS (Invitrogen, USA), 1% Penicillin-Streptomycin (Gibco, USA), 1% Glutamax (Gibco, USA), 1 mM sodium pyruvate (Sigma Aldrich, USA), 100 μM β-mercaptoethanol (Sigma Aldrich, USA), supplemented with 25 ng/ml FGF4 and 1 mg/ml heparin. Differentiation was induced by withdrawal of FGF4, Heparin and conditioned medium (Tanaka, 2006).

Full length NOSTRIN cDNA or SH3 domain deleted NOSTRIN cDNA expressed in CAG vector have been described before (Chakraborty and Ain, 2017) and were used for over-expression of NOSTRIN. TSCs were transfected using Lipofectamine LTX and Plus reagent (Invitrogen, USA).Control cells were transfected with empty vector backbone. Differentiation was induced after 6 h of transfection. Transfected cells were incubated for 60 h (Day 2.5) before RNA isolation. To confirm NOSTRIN over-expression, mRNA and protein levels were assessed by real time PCR and western blotting, respectively.

2.3. RNA isolation and quantitative real time PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen, USA). TSCs were trypsinized and differentiated trophoblast cells were scraped before lysis. RNA was reverse transcribed using SuperScript III Reverse Transcription kit (Invitrogen, USA). Ten-fold dilution of cDNAs, Power SYBR GREEN PCR Master Mix (ABI, USA) was used for each PCR reaction and run using a 7500 Real-Time PCR System (ABI, USA). Conditions used included holding stage (95 °C for 10 min) and 40 cycles (95 °C for 15 s and 60 °C for 1 min) followed by a dissociation stage (95 °C for 15 s, 60 °C for 1 min, and then 95 °C for 30 s). Primers used for real time PCR are enlisted in Table 1. Samples were normalized to rpL7. At least three different biological replicates were used.

2.4. Western blot analysis, cell fractionation and immunoprecipitation

Western blot analysis was done as described before (Chakraborty et al., 2018). For TSCs, cells were trypsinized using 0.05% Trypsin (Gibco, USA) to get a pure population of stem cells and for differentiated trophoblast cells scraper was used before lysis. Cell fractionation was performed using a kit (9038, Cell Signaling Technology, USA) according to manufacturer's instructions.

For immunoprecipitation, cell lysates were incubated overnight with either anti-N-WASP antibody, anti-cdc2 or control isotype-matched IgG 4 °C to allow formation of the antigen-antibody complex. PureProteome Protein A/G Mix Magnetic Beads (Millipore, USA) were used to capture this complex and eluted under denaturing conditions as per manufacturer's protocol.

2.4.1. Antibodies

Anti-NOSTRIN antibody (ab116374) and anti-Rpl7 (A300 -741A) were purchased from Abcam (USA) and Bethyl laboratories (USA), respectively. Anti-Dynamin (2342) and anti-N-WASP (4848) anti-cdc2 (77055), anti-phospho-cdc2-T14 (2543), anti-phospho-cdc2-Y15 (4539), HRP conjugated goat anti-rabbit (7074) antibodies were purchased from Cell Signaling Technology (USA). Anti Histone H3 (sc-8654) and donkey anti-goat IgG (sc-2020) were obtained from Santa Cruz Biotechnology (USA).

2.5. In situ hybridization

In situ hybridization was performed using digoxigenin labeled antisense and sense probes as previously described (Braissant and Wahli, 1998; Saha et al., 2017).

2.6. Immunofluorescence

Trophoblast cells were harvested on day 6 of differentiation. Cells were fixed using 4% paraformaldehyde, washed with PBS, blocked with PBS containing 5% goat serum and 0.3% TritonX followed by incubation with anti-NOSTRIN (ab116374, Abcam, USA) antibody (1:20) in PBS containing 1% BSA and 0.3% TritonX for 2 h. Following washing

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