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Title: **Balanced levels of nerve growth factor are required for normal pregnancy progression**

Running head: **Disturbances in NGF levels compromise pregnancy**

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Abstract

Nerve growth factor, the first identified member of the family of neurotrophins, is thought to play a critical role in initiating the decidual response in stress-challenged mouse pregnancies. However, the contribution of this pathway to physiological events during the establishment and maintenance of pregnancy remains largely elusive. Using alternatively NGF depletion and supplementation strategies, we here show that successful mouse pregnancy is sensitive to disturbances in NGF concentrations. Administration of NGF further boosted fetal loss rates in the high abortion CBA/J x DBA/J mouse model by amplifying a local inflammatory response through recruitment of NGF-expressing immune cells, increased decidual innervation with substance P^+ fibers and a Th1 cytokine shift. Likewise, treatment with an NGF neutralizing antibody in BALB/c mated CBA/J mice, a normal pregnancy model, also induced abortions associated with increased infiltration of tropomyosin kinase receptor A expressing NK cells to the decidua. Importantly, in neither of the models pregnancy loss was linked to defective ovarian function, angiogenesis or placental development. We further demonstrate that spontaneous abortion in humans is associated with up-regulated synthesis and an aberrant distribution of NGF in placental tissue. Thus, a local threshold of NGF expression seems to be necessary to ensure maternal tolerance in healthy pregnancies, but when surpassed may result in fetal rejection due to exacerbated inflammation.

Introduction

Neurotrophins (NTs) are a family of peptide growth factors sharing structure homology and physiological function which are essential for the development of the mammalian nervous system by virtue of their trophic effects on neuronal cells (Lindsay, et al. 1994). Among NTs, nerve growth factor (NGF) plays a pivotal role controlling the differentiation and survival of peripheral sympathetic and sensory nerve fibers as well as the functionality of cholinergic neurons (Aloe, et al. 2012, Lindsay, et al. 1994). The mature NGF molecule results from proteolysis of a precursor form (proNGF), which is also bioactive and exerts both pro-apoptotic and neurotrophic effects during development and adult life (Fahnestock, et al. 2001, Fahnestock, et al. 2004). Both forms exert their biological activities upon ligation of the specific tropomyosin kinase receptor A (TRKA), which is a typical tyrosine kinase receptor (Huang and Reichardt 2003); as well as of the low-affinity and non-selective p75 pan-neurotrophin receptor (p75NTR). Neurotrophic effects of NGF boosted upon cobinding of TRKA and p75NTR, whereas the latter receptor has been found to promote apoptosis especially when bound to proNGF (Friedman and Greene 1999, Schor 2005).

Originally studied in neuronal cells, it has now become evident that NTs exert important functions in a variety of tissues including the endocrine, immune and reproductive systems (Tessarollo 1998). Pleiotropic effects of this pathway include, for instance, the control of foliculogenesis and ovarian function (Chaves, et al. 2013) and the regulation of physiological and pathological angiogenesis through interactions with the vascular endothelial growth factor (VEGF) system (Hansen-Algenstaedt, et al. 2006, Nico, et al. 2008). Additionally, studies analysing the expression profile of NGF and its receptors at the fetal-maternal interface point out to a pivotal role of this pathway in the establishment of balanced immune-endocrine interactions during pregnancy. The most important insights on this role arise from studies in mice, in which NGF expression occurs mainly in decidual tissue, peaking at early post-implantation stages (i.e., E7.5) and declining thereafter (Kanai-Azuma, et al. 1997). Interestingly, decidual NGF and TRKA expression is markedly up-regulated in the CBA/J x DBA/2J model of stress-induced immunological abortion (Tometten, et al. 2004), and the detrimental

effects of stress exposure during early pregnancy can be abolished in these mice by specific blocking of NGF signalling with an anti-NGF antibody (Tometten, et al. 2006). This local increase of NGF in stress-challenged pregnancies is associated with neurogenic inflammation involving two stages: i) stress exposure is translated in the increase of local NGF production and release of inflammatory neuropeptides (i.e. substance P, SP) from decidual sensory nerves, enhancing leukocyte trafficking and ii) NGF levels are amplified through the recruitment of NGF-producing immune cells, promoting an increase in SP^+ nerve fibers and ultimately leading to an inflammatory environment characterized by up-regulation of Th1 cytokines and adhesion molecules which causes fetal resorption (Tometten, et al. 2006).

Besides its role mediating the stress response to disrupt pregnancy maintenance, little information is currently available on the influence of NGF signalling in physiological events at the maternal-fetal interface. In mice, maximal levels of NGF expression are detected on E7.5 coinciding with the onset of placentation, and it was indeed demonstrated that NGF could promote the differentiation of trophoblast giant cells *in vitro* presumably by a p75NTR-mediated mechanism (Kanai-Azuma, et al. 1997). A similar role may be anticipated in humans, since expression of NGF peptide is detected both in the decidua and the placenta, localizing to syncytiotrophoblast cells, the chorionic mesoderm and maternal endothelial cells (Toti, et al. 2006). Thus, controlled NGF expression at the fetal-maternal interface seems to be important for physiological events such as decidualization and placentation that determine successful pregnancy outcomes. To investigate this hypothesis, we analysed the effects of either NGF administration or deprivation in two mouse pregnancy models and assessed NGF expression in human normal pregnancy and spontaneous abortion patients. Our data suggest that disturbances in NGF concentrations at the fetal-maternal interface can compromise the maintenance of healthy pregnancies.

Materials and Methods

Animals

Mice (6 to 8 weeks old) were purchased from Charles River (Sulzfeld, Germany) and maintained in a barrier animal facility with a 12 h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to requirements of the state authority for animal research conduct (LaGeSo, G0134/07, Berlin). In this study, two animal models were used: 1) normal allogeneic pregnancy CBA/J females mated with BALB/c males and 2) a high abortion rate mouse model DBA/2J mated CBA/J female mice. The presence of a plug was designated as embryonic day (E) 0.5.

NGF treatment

DBA/2J or BALB/c mated CBA/J female mice were treated with NGF (20µg/mouse/day, Sigma Aldrich, Germany; (Joachim, et al. 2007)) administered i.p. on E5.5 and 6.5. On E7.5 and 13.5 mice from the respective groups (n=6 animals/E) were sacrificed and uterine tissue from whole implantation sites was processed for histological sectioning. In addition, some of the tissues on E7.5 were used for isolation of different leukocytes subsets. Gestation day matched control animals were treated likewise receiving single i.p. injections of vehicle (phosphate buffered saline , PBS).

NGF neutralization

After overnight cohabitation with BALB/c males, CBA/J females with vaginal plugs (E0.5) were segregated and randomized to two different treatment groups. The control group (n=6) received i.p. injections of 200 µl non-immune rabbit serum (3.2µg/Kg BW, Sigma Aldrich, Germany) in PBS from E2.5 to 6.5. A second group (n=6) was injected i.p. with neutralizing antiserum against NGF (3.2 µg/kg BW, Sigma Aldrich, Germany) daily between E2.5 and 6.5 as previously described (Tometten, et al. 2006). On E7.5 and 13.5 mice from the respective groups (n=6/E) were sacrificed and uterine tissue

131 from the implantation sites was processed for histological sectioning. In addition, some of the tissues
132 on E7.5 were used for isolation of different leukocytes subsets.
133
134 ***Fetal resorption rate***
135 Mice sacrificed on E13.5 were analysed and the total number of implantations and resorption sites (=
136 abortions) were recorded. The resorption sites were identified by their small size and necrotic
137 hemorrhagic appearance compared to normal embryos and placentas. The fetal resorption rate was
138 calculated as the ratio of resorption sites and total implantation sites (resorptions + normal
139 implantation sites), as described previously (Tometten, et al. 2006) .
140
141 ***Study patients***
142 For the analysis of NGF, TRKA, p75NTR expression, placental tissue was obtained from patients
143 undergoing elective termination of pregnancy during the first trimester (8–12 weeks of gestation, NP
144 samples) and from spontaneous abortions (SA samples). Characteristics of the recruited participants
145 are summarized in (Table 1). Samples were processed immediately after collection for the isolation of
146 trophoblast cells and histological sectioning. Informed written consent was obtained from all patients
147 before their inclusion in the study, which was approved by the local ethics committees of Geneva
148 University Hospital.
149
150 ***Histology***
151 For histological analysis, ovaries on E7.5 and whole implantations on E13.5 were fixed with 10%
152 buffered formalin, dehydrated in ethanol, embedded with paraffin, and stained following
153 Hematoxylin and Eosin (H&E) protocol. Briefly, samples were washed 5 min in TBS buffer followed by
154 incubation in Mayer´s Haematoxylin for 12 min at room temperature (RT). Slides were then washed
155 in tap water for 15min and incubated in Eosin for 20min. This was followed by dehydration through
156 ethanol 100% (2 times, 2min each) and xylene (2 times, 5 min each) and mounting in Vitro-Clud (R.

157 Langenbrinck, Germany). Tissue sections were examined using a light microscope (Axiophot) and
158 photographs taken with Axio Cam HRc. Photo documentation was performed using the digital image
159 analysis system Spot advanced software, version 8.6 (Visitron Systems).
160
161 ***Purification of Cytotrophoblast (CTB) and extravillous cytotrophoblast (EVT) cells***
162 Trophoblast cells were isolated by immunopurification as described previously (Tirado-Gonzalez, et
163 al. 2013). Identification of CTB was based on cytokeratin 7 positivity and absence of vimentin
164 expression. Isolated EVT were identified as cytokeratin 7 and HLA-G positive, vimentin negative cells.
165
166 ***Enzyme-Linked Immunosorbent Assay (ELISA)***
167 Serum samples from E7.5 were tested in competitive ELISA using kits obtained from R&D Systems to
168 quantify VEGF-A (Duoset mouse VEGF, cat DY493) following the manufacturer's recommendations.
169 The quantification of progesterone levels in serum were determined using rat/mouse progesterone-
170 EIA kit (DRG Diagnostics, Germany, cat EIA-5486) following the manufacturer's recommendations.
171
172 ***Endoglin staining***
173 Uterine tissue sections from E7.5 were stained following our standard protocol (Blois, et al. 2007).
174 Briefly, slides were washed 3 times in TBS for 5 min, blocked with 2% normal serum for 20 min and
175 incubated overnight at 4°C with the primary anti-endoglin Ab (1:100, Santa Cruz Biotechnology).
176 Negative controls were established by replacing the primary Ab with irrelevant IgG. After washing,
177 endoglin stained sections were incubated 1h at RT with TRITC-conjugated secondary antibodies
178 (Jackson ImmunoResearch). Sections were analyzed using a confocal laser scanning microscope (cLSM
179 510, Carl Zeiss).
180
181 ***RNA Isolation and Quantitative Real-Time PCR***

Total RNA was extracted from isolated CTB (first and third trimester) and EVT using the RNeasy mini kit (Qiagen, Germany), whereas total RNA from mouse implantation site tissues on E7.5 was extracted using the Nucleospin RNA/protein isolation kit (Macherey-Nagel). After DNase digestion (Invitrogen, Germany), cDNA was generated using random primers (Invitrogen) followed by quantitative real-time RT-PCR performed on the TaqMan 7500 System (Applied Biosystems). For each reaction, 1µL cDNA, synthesized from 1µg RNA in 25µL, was used in a total volume of 12µL containing 6.25µL of Power SYBR Green PCR mastermix (Applied Biosystems), 3.75µL DEPC water and 450nM of the appropriate forward and reverse primer. Primer sequences were as follows: *NGF* forward 5´-TGAAGCTGCAGACACTCAGG-3´; *NGF* reverse 5´-CTCCCAACACCATCACCTCC-3´; *TRKA* forward 5´-CATCGTGAAGAGTGGTCTCCG-3´; *TRKA* reverse 5´-GAGAGAGACTCCAGAGCGTTGAA-3´; *P75NTR* forward 5'-TGGGCAGGACCTCAGAGTCC-3'; *P75NTR* reverse 5'-TTCCTCCCTCTGAGTCTCTG-3'; *CYCLOPHILINA* forward 5´-TACGGGTCCTGGCATCTTGT-3´; *CYCLOPHILINA* reverse 5´-CCATTTGTGTTGGGTCCAGC-3´; *Vegf* forward 5'-ATCTTCAAGCCGTCCTGTGT-3'; *Vegf* reverse 5'-GCATTCACATCTGCTGTGCT-3', *Flt1* forward 5'-CGGAAGGAAGACAGCTCATC -3'; F*lt1* reverse 5'-CTTCACGCGACAGGTGTAGA-3'; *Hprt* forward 5'- GTTGGATACAGGCCAGACTTTGT-3' and *Hprt* reverse 5'-CACAGGACTAGAACACCTGC-3'. Relative expression of *NGF, TRKA, p75NTR, Vegf and Flt1* was calculated according to the equation Rel. Exp (RE)= 2-DCt. The obtained Ct value of each gene of interest was normalized to the Ct of the reference genes (Human: *CYCLOPHILINA*) or (Mouse: *Hprt*) as follows: Ctnorm = Ctgoi - Ctref with norm = normalized, goi = gene of interest, and ref = reference gene.

Immunofluorescence staining for NGF, TRKA and p75NTR

Cytospins or mouse and human cryostat sections (8µm) were stained using a standard immunofluorescence protocol. Primary polyclonal antibodies [anti- NGF (cat. sc-549; 1:100), anti-TRKA (cat. sc-118; 1:100) and anti-p75NTR (cat. sc-5634; 1:100) acquired from Santa Cruz, Biotechnology, Germany] were incubated overnight at 4° C in a humidity chamber, after which

binding was detected using a rhodamine-labeled secondary antibody (Dianova, Hamburg, Germany; 1:200). Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). After washing, all sections were mounted and stored at -20°C until analyzed. Negative controls in which the primary antibody was replaced with irrelevant goat IgG showed no specific immunoreactivity. Sections were examined by two independent persons blinded with regard to the treatment of the mice at x400 magnification under a Zeiss Axioscope fluorescence microscope. Photo documentation was performed using digital image analysis system (Spot advanced software, version 3.5.2; Visitron Systems; Puchheim, Germany).

NGF and TRKA immunohistochemistry in Human specimens

Sections of paraffin-embedded tissue (n=16 normal pregnancy and n=15 spontaneous abortion) were cut at 4µm, deparaffinised, rehydratated and washed in Tris-buffered saline (TBS), followed by blocking of endogenous peroxidase through incubation with 3% H_2O_2 in methanol for 30 min at RT. After incubation with 2% normal serum for 20 min, rabbit anti-human NGF or TRKA IgG (1:200, Santa Cruz Biotechnology, Heidelberg, Germany) were incubated overnight (ON) at 4°C. The slides were then washed and incubated with goat anti-rabbit HRP-conjugated secondary Ab (1:200, Jackson ImmunoResearch, Germany) for 1h at RT followed by detection with 3,3'-diaminobenzidine (DAB) chromogen (DAKO, Germany). After washing, nuclei were counterstained with 0.1% Mayer's hematoxylin followed by a standard dehydration procedure and mounting in Vitro-Clud medium (R. Langenbrinck, Germany). Negative controls were established by replacing the primary antibody with an equal concentration of irrelevant rabbit IgG.

SP and CGRP Staining

DBA/2J or BALB/c mated CBA/J female were perfusion-fixed using a mixture of paraformaldehyde and picric acid (Peters, et al. 2002). SP^+ and $CGRP^+$ nerve fibers were determined in 14µm thick sections. Primary antibody binding (SP antiserum, monoclonal; Chemicon, Temecula, CA, 1:100, CGRP

antiserum, monoclonal, Chemicon, Temecula, CA) was detected by a rhodamine-labelled secondary antibody (Dianova, Hamburg, Germany, dilution, 1:200). Nuclei were counterstained with DAPI and mast cells with fluorescein-labelled streptavidin (Botchkarev, et al. 1997).

Preparation of uterine cell suspensions

In order to obtain suspensions of uterine leukocytes for NGF and TRKA characterization by immunofluorescence, a method described previously (Tometten, et al. 2006) was used. Briefly, uteri were collected, washed with sterile PBS, carefully cut into small pieces, collected in tubes containing HBSS and digested for 20 min at 37°C under slight agitation with 200 U/ml hyaluronidase, 1 mg/ml collagenase, 1 mg/ml BSA/fraction V (all Sigma, Germany) and 0.2 mg/ml DNase I (Boehringer Mannheim GmbH, Germany). The isolated cells were then collected in a fresh tube through a 100 µm net (Becton Dickinson, San Francisco, USA) and washed with RPMI 1640 containing 10% fetal bovine serum (FBS). The procedure was repeated twice, with HBSS medium containing no cocktail of enzymes. Individual leukocyte populations were isolated using Miltenyi Biotec immunomagnetic kits ($CD45^+$, $CD4^+$, $CD8^+$, $CD11c^+$ or $CD49b^+$).

NGF stimulation in vitro

Leukocyte subsets from uterine cell suspensions from BALB/c or DBA/2J mated CBA/J females obtained on E8.5 were seeded (2×10^5 cells per well) in 96-well plates and stimulated with NGF (0, 10 and 20ng/ml, Sigma Aldrich, Germany) for 48h. Cultures were performed at 37°C in a 5% CO2 atmosphere in RPMI 1640 supplemented with antibiotic (50U/ml penicillin and 50 µg/ml streptomycin), 2g/L sodium bicarbonate, 2mM L-glutamine, 1mM pyruvate and 10% fetal calf serum (FCS). Supernatants were stored at -80°C until cytokines analysis by cytometric bead array (CBA).

Cytokine determination

Cytokines (TNFA, IFNG, IL6 and IL10) were analyzed in cell culture supernatants using cytometric bead arrays (BD Biosciences, Heidelberg, Germany) as previously described (Blois, et al. 2007).

Statistical analysis

The number of animals included in each experimental group was indicated accordingly. Data are presented as median from three replicate experiments. Statistical significance was determined using the nonparametric Mann-Whitney U test, with a P value of less than 0.05 being considered as significant. Statistical analysis was carried out with GraphPad Prim 5.0 (GraphPad Software Inc.).

Results

NGF administration during early pregnancy boosts spontaneous abortion rates in the CBA/J x DBA/J mouse model

In the CBA/J x DBA/J mating combination, exposure to stress (i.e., to sonic stimulation) on E5.5 provokes a spontaneous abortion syndrome previously shown to be associated with up-regulation of NGF signalling and features of neurogenic inflammation (Tometten, et al. 2006, Tometten, et al. 2004). Thus, our first aim was to analyse the effect of NGF treatment during early stages of pregnancy (i.e., E5.5) in DBA/J mated CBA/J female mice (Fig. 1A). As shown in Fig 1B, NGF treated females displayed significantly increased abortion rates with respect to controls, accompanied by a reduction in the number of total implantation sites as evidenced on E13.5. By contrast, administration of NGF to BALB/c mated CBA/J mice (which represent a normal allogeneic pregnancy model) caused no alterations in the frequency of abortions and the number of implantations compared to control untreated mice (Fig. 1C).

Interestingly, NGF induced abortions in CBA/J x DBA/J mice were apparently not linked to dysregulated angiogenic growth factor expression during the peri-implantation period, as local

expression of VEGF/Flt1, serum VEGF levels (Fig. 1D) and the distribution of the endothelial activation marker endoglin (Fig. S1A) recorded on E7.5 were similar in NGF treated and control mice. Likewise, no signs of defective ovarian function were apparent upon administration of NGF, with similar progesterone levels and ovarian histology in control and NGF treated mice on E7.5 (Fig. S1B). Histological analysis of E13.5 implantation sites further showed that administration of NGF did not cause significant alterations in placental structure (Fig. S1C).

We next evaluated decidual immune cell subsets to further investigate if, as reported for stress-challenged CBA/J x DBA/J mice, NGF induced abortions were related to local neurogenic inflammation. As shown in Fig. 1E, NGF treatment led to a significant increase in the frequency of decidual lymphocytes expressing NGF ($CD45^+$ NGF^+ cells) on E7.5, particularly of the $CD8^+$ NGF^+ (Fig. 1E, right panel) and $CD4^+$ NGF^+ T cell subsets (Fig. S1D, left panel). In contrast, no differences were observed in the abundance of decidual NGF-expressing DC ($CD11c^+$) and NK cells ($CD49b^+$) (Fig. S1D, middle and right panels). Furthermore, treatment with NGF led to a significant up-regulation of TRKA expression in decidual lymphocytes (both $CD4^+$ and $CD8^+$ cells, Fig. S1E). Regarding NGF receptor expression, increased TRKA levels were observed on decidual DC, NK cells and $CD45^+$ lymphocytes from NGF-treated mice (Fig. 1F, right panel), whereas p75NTR expression levels in the decidua did not differ from controls (Fig. S1F).

To gain insight into the mechanisms involved in the pathogenesis of NGF induced abortions, we next analysed the profile of Th1/Th2 cytokines secreted by uterine cells upon NGF stimulation *in vitro*. As displayed in Fig. 1G, uterine cells secreted significantly increased levels of IFNG and IL6 in response to NGF, whereas levels of TNFA showed no differences with respect to control cells (Fig. 1G, left and middle panels). In contrast, stimulation with NGF led to a dose-dependent decrease in the secretion of Th2 IL10 by isolated uterine cells (Fig. 1G, right panel). This shift towards Th1 cytokines was associated with signs of neurogenic inflammation in the decidua of NGF treated females, namely an increased density of SP^+ nerve fibers (Fig. 1H, left panels) and increased percentage of degranulated mast cells (Fig. S1G) compared to controls. Strikingly, the density of sensory nerve fibers expressing

calcitonin gene related peptide (CGRP), which mediates vasodilatory effects during stress-induced neurogenic inflammation (Joachim, et al. 2007), was significantly decreased in the uterus of NGF treated female mice (Fig. 1H, right panels).

Neutralizing NGF disrupts normal pregnancy progression in low-abortion mating combinations

Based on the above mentioned findings and previous results showing that NGF neutralization prevents stress-triggered abortions in the CBA/J x DBA/2J model (Tometten, et al. 2006), we next aimed to investigate the physiological role NGF plays in the maintenance of pregnancy. We therefore examined the effects of a NGF-neutralizing antibody administered to BALB/c mated CBA/J female mice (Fig. 2A). In this model, a four-day course of anti-NGF administration showed no effect in the number of total implantations registered on E13.5, but led to a significant up-regulation of the abortion rate with respect to isotype-control injected female mice (Fig. 2B). Abortions triggered by NGF neutralization were not related to differences in ovarian histology or systemic progesterone levels on E7.5 (Fig S2A), nor with significant disturbances in the expression of pro-angiogenic VEGF/Flt1 (Fig. 2C) and endoglin (Fig. S2B). Furthermore, placental structure (as analysed on haematoxylin-eosin stained sections) on E13.5 was not altered upon treatment with the anti-NGF (Fig. S2C).

Immunofluorescence analysis of sorted decidual immune cells on E7.5 revealed no differences in the frequency of total $CD45^+$, $CD4^+$, $CD8^+$ and NK cells expressing NGF (Fig. S2D), but a significant down-regulation of $CD11c^+NGF^+$ DC was observed in response to NGF neutralization (Fig. 2D). Additionally, anti-NGF treated females displayed an increased frequency of $TRKA^+$ decidual NK cells (Fig. 2E), whereas expression of this receptor in other immune cell subsets analysed (i.e., lymphocytes, DC, Fig S2E) as well as that of p75NTR (Fig. S2F) did not differ from controls.

When analysing the profile of Th1/Th2 cytokines secreted by uterine cells isolated from control DBA/2J mated CBA/J females, no differences were observed in the levels of IL6 and IFNG in response to NGF *in vitro* (Fig. 2F). However, a striking finding was that NGF induced a shift towards a Th2

response on isolated uterine cells, namely a dose dependent decrease in TNFA secretion (Fig 2F, left panel) and significantly increased levels of IL10 (Fig. 2F, right panel). In addition, anti-NGF treated females displayed an increased density of decidual SP^+ nerve fibers (Figure 2G, left panels) whereas the frequency of $CGRP^+$ fibers and degranulated mast cells (Fig S2G) were decreased with respect to controls.

Human spontaneous abortion is associated with increased NGF expression at the fetal-maternal interface

Knowing that the decidua and placental trophoblasts are a source of NGF synthesis throughout human pregnancy (Toti, et al. 2006), we next aimed at analysing the expression levels of *NGF* and *TRKA* on isolated trophoblast cells. As shown in Fig. 3A, expression of *NGF* was detected both on CTB and EVT isolated during the first trimester and also at term. During the first trimester, *NGF* expression in the CTB was significantly higher than in EVT (*P<0.01*); remaining at similar levels at term. In contrast, no differences were observed regarding the expression of the *TRKA* (Fig. 3B) or *P75NTR* (Fig. 3C) receptors, which exhibited high mRNA levels in EVT and CTB both during the first trimester and at term. To further dissect the association between the NGF pathway and pregnancy outcome, we next assessed the expression of *NGF* and its receptors in choriodecidual samples from spontaneous abortion (SA) patients and normal pregnant (NP) women. Real-time qPCR analysis showed that *NGF* levels were significantly up-regulated in SA patients respect to controls (Fig. 3D, left panel); whereas no differences were detected in the expression of *TRKA* (Fig 3D, middle panel) or the *P75NTR* receptor (Fig. 3D, right panel). In NP samples, expression of the NGF peptide as analysed by immunohistochemistry was localized mainly in decidual tissue and the CTB layer of the placenta (Fig 4A, left panels), whereas SA patients exhibited an increased immunoreactivity signal in the decidua and additional staining in the placental syncytiotrophoblast. However, both groups displayed a similar distribution pattern for TRKA expression, which localized to the decidua and the placental CTB and EVT partially overlapping NGF expression (Fig 4B). Expression of the p75NTR receptor, as

analysed by immunofluorescence, was detected on single decidual cells and on villous CTB and syncytiotrophoblasts showing a similar distribution pattern in both groups (Fig. 4C)

Discussion

The establishment of pregnancy is a complex process involving balanced interactions between the immune, endocrine and reproductive systems. We here demonstrate the physiological importance of NGF signalling in this process by showing that a normal progression of pregnancy is largely sensitive to disturbances in systemic NGF concentrations that appear to have impact on local adaptation processes that take place at the maternal-fetal interface. A variety of pleiotropic effects of the NGF pathway; including the control of ovarian function, inflammation and angiogenesis, are most likely to influence the outcome of pregnancy (Tometten, et al. 2005). In particular, decreased progesterone levels in diestrus and an impaired response to hCG-like activity have been observed upon NGF overexpression in mouse ovaries (Dissen, et al. 2009). However, results from our mouse studies showed no overt defects in progesterone levels and luteal structure, suggesting that disruption of pregnancy caused by deregulation of NGF levels does not result from alterations in ovarian physiology. The lack of effects in NGF treated mice may obey to the doses, route and time frame of administration in our study, as treatment was conducted post-ovulation (E1.5) which is one of the main events in the ovarian cycle influenced by this pathway (Dissen, et al. 2000).

Our results showed that disruption of the NGF pathway (i.e., by treatment with an anti-NGF Ab) *in vivo* at early post-implantation stages induced abortion, which is consistent with a protective role of this NT in the context of a normal pregnancy. Since effects on ovarian function, decidual angiogenesis and placental morphology were ruled out in this study, such a requirement for NGF may most probably be related to the reported immunomodulatory properties of this molecule, namely in the regulation of T cell responses (Aloe, et al. 1999). Indeed, our *in vitro* findings showed that NGF is able to promote a Th2 cytokine shift (i.e., decreased TNF-α and increased secretion of IL10) on isolated

uterine lymphocytes, which is consistent with previous studies demonstrating a selective expression of NGF and TRKA in Th2 cells (Arredondo, et al. 2001, Sekimoto, et al. 2003). Thus, physiological levels of NGF may function to support Th2 cells and suppress Th1 function at the fetal-maternal interface modulating a cytokine environment compatible with pregnancy maintenance.

Interestingly, we also found an increased frequency of $TRKA^+$ decidual NK cells and decreased NGF-expressing $CD11c^+$DC following NGF neutralization, which may imply that the functions of these innate immune subsets are also deregulated in the absence of NGF signaling at the maternal interface. Among other functions, decidual NK cells are of utmost importance for the control of trophoblast invasion, vascular remodeling and immune tolerance at the maternal-fetal interface (Ashkar, et al. 2000, Fu, et al. 2013, Gonzalez, et al. 2012). Recent studies have demonstrated that TRKA expression is dynamically regulated on mouse NK cell subsets and is further enhanced upon activation, whereas NGF has been shown to act as a negative modulator of NK cell degranulation (Ralainirina, et al. 2010). Thus, it is conceivable that the up-regulated frequency of $TRKA^+$ NK cells observed in our study represents an aberrant activation of this subset in the context of NGF deprivation at the fetal-maternal interface. Since NK cell derived signals have been shown to be important for the control of immunogenic activation of DC (Gonzalez, et al. 2012), aberrant NK cell activation upon neutralization of NGF is also likely to influence DC functions at the fetal-maternal interface. DC in turn are known regulators of NK cell differentiation and function at the uterine lining (Karsten, et al. 2009, Krey, et al. 2008), and have been shown to increase their NGF expression in response to immunogenic maturation signals (i.e., LPS) (Jiang, et al. 2008). In this context, it is tempting to speculate that physiological levels of NGF may be necessary for the establishment of an effective immunoregulation (i.e. cooperation between DC and NK cells) at the early fetal-maternal interface. Though we found no overt defects in angiogenic growth factor expression at E7.5 or placental structure at E13.5, it cannot be completely discarded that direct effects of the NGF pathway in the control of developmental processes occurring post-implantation (i.e., decidualization and placentation) contribute to the increased abortion rates observed following NGF neutralization.

Indeed, such a role has already been demonstrated in mice, where decidual derived NGF functions as a growth factor promoting promoting the differentiation of trophoblast giant cells (Kanai-Azuma, et al. 1997), which constitute one of the main sources of pro-angiogenic factors during mouse placentation (Hemberger, et al. 2003). Evidence on a similar role in human placentation is still elusive, but may be anticipated based on previous studies reporting NGF expression in the decidua and the placenta during the first trimester (Toti, et al. 2006). Indeed, we here showed a differential expression of NGF on CTB and EVT cells isolated from normal first trimester placental tissue, which may be related to possible autocrine/paracrine effects of NGF in the control of trophoblast lineage differentiation. A thorough examination of the influence of the NGF and other NT mediated pathways in trophoblast cells isolated from human placentas would greatly improve our understanding of their association with pregnancy complications.

On the other hand, we found that exposure to supraphysiological levels of NGF during the early post-implantation period (i.e., in NGF treated female mice) also induced a spontaneous abortion syndrome, with features resembling those observed in stress-challenged pregnant mice. Typical signs of neurogenic inflammation were observed in such NGF-treated mice including increased infiltration of NGF-producing $CD4^+$ and $CD8^+$ T cells, increased innervation with SP^+ fibers, enhanced mast cell degranulation and a Th1 cytokine shift in decidual lymphocytes characterized by increased secretion of IL6 and IFNG and decreased IL10. Thus, in a manner similar to stress-triggered abortions (Tometten, et al. 2006), increased NGF levels at the fetal-maternal interface skew the immune system towards an inflammatory Th1 response, which is further amplified through the recruitment of NGF-expressing immune cells ultimately resulting in disruption of maternal tolerance and fetal loss. Interestingly, increased innervation with SP^+ and $CGRP^+$ fibers has been associated with the skin response to stress, provoking a typical neurogenic inflammation reaction in which SP promotes immune cell recruitment further amplified by vasodilatory effects of CGRP (Joachim, et al. 2007). Our finding that only SP^+ innervation and not the density of $CGRP^+$ fibers was increased upon NGF

treatment may imply that the inflammatory response causing fetal rejection is maintained and prolonged by other yet unknown mechanisms instead of CGRP- mediated vasodilation. For instance, our previous studies have shown that the decidual up-regulation of adhesion molecules ICAM1 and P-selectin in response to stress was abrogated in NGF-neutralized mice (Tometten, et al. 2006), suggesting that stress- and NGF-induced abortions are dependent on adhesion molecule mediated inflammatory pathways. Accordingly, we found a significant increase in NGF synthesis at the fetal-maternal interface of spontaneous abortion patients accompanied by up-regulated NGF expression in the placental syncytiotrophoblast with respect to controls. While it remains to be determined whether NGF deregulation is causally linked to human spontaneous abortions, these findings agree well with our previous studies demonstrating an up-regulation of decidual NGF expression and a pregnancy protective effect of anti-NGF treatment in stress-challenged mice (Tometten, et al. 2006, Tometten, et al. 2004). Interestingly, the beneficial effects of anti-NGF therapy were abrogated in animals treated with a high antibody dose, in which the fetal loss rates were further boosted with respect to stressed mice (Tometten, et al. 2006) suggesting that NGF deprivation beyond a certain threshold also compromises pregnancy maintenance. Taken together, our results imply that healthy gestations are dependent on a balanced expression of NGF to ensure adequate maternal immunemodulation and developmental processes at the fetal-maternal interface. We anticipate that these results may have important implications for the understanding of human pregnancy complications related to immunological disbalances such as spontaneous abortion.

Author contributions

M.T. and S.M.B. designed research; P.F., G.B., I.T-G., M.C., M.T. and S.M.B. performed research; P.F., G.B., and I.T-G analyzed data; P.M. assisted research; E.M.P., B.F.K. and M.R. gave input on writing the manuscript; G.B. and S.M.B. wrote the manuscript.

Declaration of interest

The authors declare that no conflicts of interest exist.

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Figure legends

Figure 1. ***NGF treatment during early post-implantation stages disrupts pregnancy maintenance in the abortion prone CBA/J x DBA/2J mating combination.*** (A) Experimental design: DBA/2J mated CBA/J females received two consecutive doses of NGF starting at E5.5, as stated in Methods. Females were sacrificed on E7.5 for the analysis of decidual immune cells and in vitro experiments and on E13.5 for assessment of fetal loss rates and total implantations. (B) Fetal loss rates (calculated as R/V+R, where R=resorptions and V= viable implants; left panel) and total number of implantations (right) observed in NGF treated mice. Fetal loss rates were significantly increased in response to NGF treatment. (C) Summary of the experimental design and results for the assessment of effects of NGF supplementation in the CBA/J x BALB/c mouse pregnancy model. NGF treated females showed no differences in fetal loss rates (middle panel) and total number of implantation sites (right panel) recorded on E13.5 respect to controls. (D) Evaluation of the angiogenic status in DBA/J mated CBA/J females upon treatment with recombinant NGF during early stages of pregnancy. Circulating levels of free VEGF (left) and decidual mRNA levels of *Vegf* (middle) and *Flt1* (right panel) analysed on E7.5 showed no differences compared to control mice. (E) Immunofluorescence analysis of NGF-expression on sorted decidual immune cells isolated at E7.5. Representative cytospins are displayed for $CD45^+NGF^+$ cells (left panel) and $CD8^+$ NGF^+ cells (right). (F) Quantification of TRKA and p75NTR expression on immune cell subsets and decidual cells isolated on E7.5, as analysed by immunofluorescence. NGF induced abortions were associated with a significant increase in $CD45^+$,

593 $CD11c^+$ and $CD49b^+$ cells expressing TRKA (left), whereas no differences were observed in decidual
594 p75NTR expression (right panel). (G) Th1 and Th2 cytokine secretion by isolated uterine leukocytes in
595 response to NGF. No differences were observed in TNFA levels (left panel), but NGF significantly
596 increased IFNG and IL6 (middle panels) and decreased levels of the Th2 cytokine IL10 (right panel).
597 (H) Immunofluorescence analysis of SP (left panels) and CGRP (right panels) expression at the
598 decidua on E7.5. NGF treated mice displayed increased innervation with SP^+ fibers, whereas the
599 density of $CGRP^+$ fibers was decreased. In all figures, * and ** denote *p<0.05* and *p<0.001* as
600 assessed by the Mann-Whitney U test.
601
602 **Figure 2.** ***NGF neutralization induces abortion in the CBA/J x BALB/c mating combination.*** (A)
603 Experimental design: BALB/c mated CBA/J females with vaginal plugs were treated daily with a
604 neutralizing NGF antibody starting at E2.5, as detailed in Methods. Females were sacrificed on E7.5
605 for the analysis of decidual immune cells and in vitro experiments and on E13.5 for assessment of
606 fetal loss rates. (B) Fetal loss rates (left) and total number of implantations (right panel) observed in
607 response to NGF neutralization. Treatment with anti-NGF significantly increased the fetal loss rate in
608 BALB/c mated CBA/J mice. (C) Evaluation of the systemic and local angiogenic status in anti-NGF
609 treated CBA/J female mice. Levels of free VEGF in serum (left) and decidual *Vegf* and *Flt1* mRNA
610 (middle and right panels) on E7.5 did not differ from those recorded in isotype-treated control mice.
611 (D) Analysis of NGF expression, as assessed by IF on sorted decidual immune cells at E7.5.
612 Representative cytospins are displayed for NGF^+ DC ($CD11c^+$ cells, left panel), which were significantly
613 increased upon NGF neutralization. (E) Summary of IF analysis of decidual TRKA (left) and p75NTR
614 expression (right panel) recorded on E7.5 in anti-NGF treated female mice. Neutralization of NGF led
615 to a significant increase in decidual $TRKA^+$ NK cells ($CD49b^+$ cells, left panel) respect to controls,
616 whereas no differences were observed in the expression of p75NTR. (F) Th1 and Th2 cytokine
617 secretion by NGF-stimulated uterine leukocytes in vitro. NGF caused a Th2 shift by significantly
618 decreasing TNFA levels (left panel) and enhancing IL10 secretion (right panel). (G) Decidual SP (left

619 panels) and CGRP (right panels) expression, as analysed by IF on E7.5. Anti-NGF treated mice showed
620 a reduced density of SP^+ nerve fibers, whereas no differences were observed regarding $CGRP^+$
621 innervation. In all figures, * and ** denote *p<0.05* and *p<0.001* as assessed by the Mann-Whitney U
622 test.
623
624 ***Figure 3. Human spontaneous abortion is associated with increased NGF expression at the fetal-***
625 ***maternal interface.*** (A) Real-time PCR analysis of *NGF* on isolated trophoblast cells during normal
626 human pregnancy. First trimester CTB express significantly increased levels of NGF compared to EVT,
627 and this expression remains high at term. (B) *TRKA* expression on isolated trophoblast from normal
628 pregnancy, as analysed by qPCR. No differences were observed between expression levels at the first
629 trimester and at term, or between the different trophoblast lineages analysed. (C) Quantification of
630 *P75NTR* mRNA levels expressed by normal trophoblast cells isolated during the first trimester and at
631 term. Villous CTB expression levels of *P75NTR*, as assessed by qPCR, remained unaltered throughout
632 pregnancy and showed no differences compared to first trimester EVT. (D) qPCR analysis of *NGF* (left
633 panel), *TRKA (middle) and P75NTR* (right) expression in normal pregnancy and spontaneous abortion
634 patients. Human spontaneous abortion is characterized by increased choriodecidual expression of
635 *NGF* mRNA, whereas no differences were observed in the *TRKA* and *P75NTR* receptors. In all figures,
636 * and ** denote *p<0.05* and *p<0.001* as assessed by the Mann-Whitney U test.
637
638 ***Figure 4. An aberrant pattern of NGF expression at the maternal-fetal interface characterizes***
639 ***human spontaneous abortions***. (A) Immunohistochemical analysis of NGF in choriodecidual biopsies
640 obtained from normal pregnancy (NP, left) and spontaneous abortion patients (SA, right panels). NGF
641 expression was localized mainly in decidual tissue (upper panels) and the CTB and EVT layers of NP
642 placentas (lower panels), whereas SA samples showed additional staining in the syncytiotrophoblast.
643 (B) Tissue distribution of TRKA expression at the maternal fetal interface, as analysed by IHC in
644 choriodecidual samples of normal pregnancies (left) and spontaneous abortions (right panels). Both

groups displayed a similar distribution pattern for TRKA expression, localizing to the decidua and the CTB and EVT. (C) Immunofluorescence analysis of p75NTR expression in choriodecidual biopsies obtained from NP and SA patients. In both groups, p75NTR was detected on single decidual cells (upper panels) and strongly staining the placental CTB and syncytium (lower panels).

Figure S1. ***NGF treatment boosts the abortion rate in the CBA/J x DBA/2J combination.*** (A) Representative examples of IF stainings for endoglin (red) on E7.5 implantation sites from NGF-treated and control mice. Endoglin showed a similar distribution in both groups, localizing to endothelial cells of the vascular zone (VZ) and mesometrial decidua (MD) adjacent to the embryonic cavity (E). (B) Evaluation of the effect of NGF supplementation on serum progesterone levels (left) and ovarian histology (right panels) in DBA/J mated CBA/J female mice. NGF treatment did not produce significant alterations in ovarian functions on E7.5, as both groups displayed similar progesterone levels and a normal ovarian structure, with multiple follicular images and corpora lutea (CL). (C) Representative pictures of H&E stained whole E13.5 implantation sites, showing that normal placental structure was conserved upon NGF administration in the CBA/J x DBA/J model. Abbreviations: PL, placenta; DB, decidua basalis; F, fetus. (D) Immunofluorescence analysis of NGF expression on sorted decidual immune cells isolated at E7.5. $CD4^+$ NGF^+ cells showed a significantly increased frequency in NGF treated mice (right panel), whereas no differences were observed on $CD11c^+$ DC and NK cells expressing NGF. (E) TRKA expression on isolated decidual leukocytes, as analysed by IF. NGF significantly increased the frequency of $CD4^+$ $TRKA^+$ and $CD8^+$ $TRKA^+$ cells on E7.5. (F) Decidual p75NTR expression, as assessed by IF on E7.5. NGF treated mice showed no differences in the distribution or expression levels of p75NTR respect to PBS treated controls. (G) Mast cell degranulation in the decidua, as analysed by IF at E7.5. The frequency of degranulated mast cells was significantly increased in NGF treated mice. Left pictures show representative examples of cytospins from NGF- (up) and PBS-treated mice (low). In all figures, * and ** denote $p<0.05$ and $p<0.001$ as assessed by the Mann-Whitney U test.

Figure S2. ***Fetal loss rates are increased in the CBA/J x BALB/c combination following NGF neutralization.*** (A) Systemic progesterone levels and ovarian histology in BALB/c mated CBA/J female mice are conserved upon NGF neutralization. Both groups displayed similar progesterone levels (as assessed by ELISA, left panel) and corpora lutea (CL) with a normal histology on E7.5, indicative of unaltered ovarian functions upon treatment with the anti-NGF. (B) Endoglin expression on E7.5 implantation sites from anti-NGF treated and control mice, as assessed by IF. Endoglin (red) showed a normal localization in both groups in endothelial cells of the vascular zone (VZ) and spreading towards the mesometrial pole (MD). (C) Histological analysis (H&E) of E13.5 whole implantation sites, showing that placental structure was unaltered upon anti-NGF treatment in the CBA/J x BALB/c model. Abbreviations: PL, placenta; DB, decidua basalis; F, fetus. (D) Immunofluorescence analysis of NGF on sorted decidual immune cells isolated at E7.5. No differences were observed in the frequency of $CD45^+$, $CD4^+$, $CD8^+$ and $CD49b^+$ cells expressing NGF with respect to controls (E) TRKA expression on isolated decidual leukocytes, as analysed by IF. Anti-NGF treated mice showed no differences in the frequency of $CD4^+$, $CD8^+$ and $CD11c^+$ cells expressing TRKA. (F) Decidual p75NTR expression, as assessed by IF on E7.5. No differences were observed in the distribution or expression levels of p75NTR between anti-NGF and isotype control treated females. (G) IF assessment of degranulated mast cells in decidual tissue at E7.5. The frequency of degranulated mast cells was significantly increased in anti-NGF treated mice. Left pictures show representative examples of cytospins from anti-NGF- treated (up) and control mice (low). In all figures, * and ** denote $p<0.05$ and $p<0.001$ as assessed by the Mann-Whitney U test.

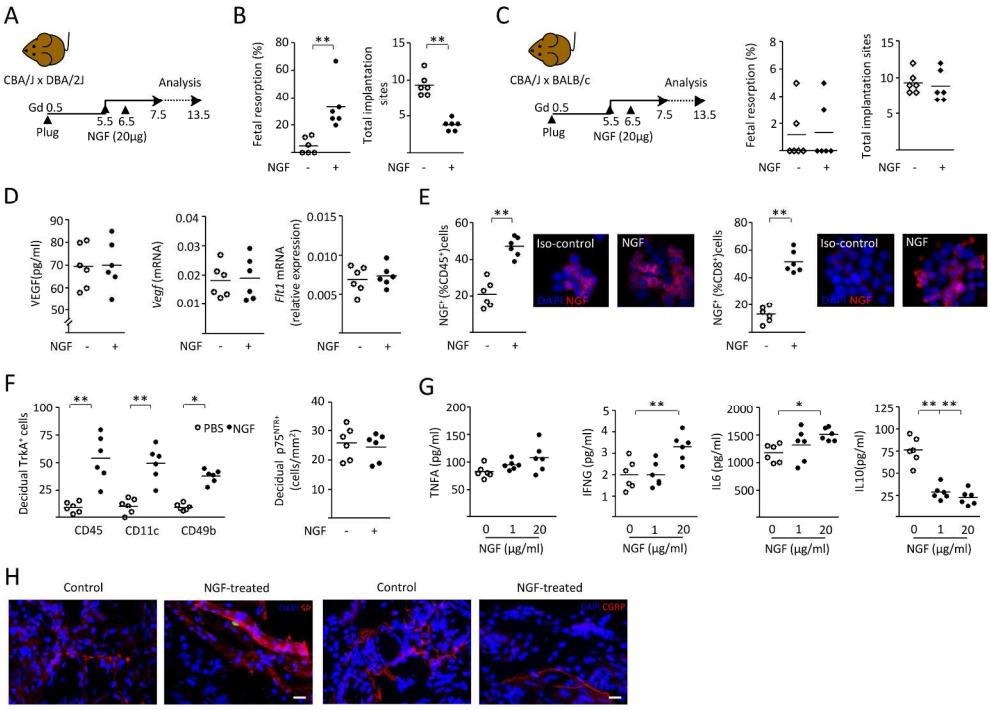
Table 1. Characteristics of the recruited participants

Parameters	Normally progressing pregnancy (n=16)	Spontaneous abortion (n=15)
Age	29.7 ± 2.80	30.5 ± 3.50
GW	8-12	8-12

Abbreviations: GW: gestational age in weeks.

Note: Inclusion criteria: week of gestation 8-12, no fertility treatment, no hepatitis B/C or HIV infection; no signs of an imminent miscarriage such as vaginal bleeding, low ßHCG, missing embryonic/fetal heart rate during ultrasound screening.

Exclusion criteria for the spontaneous abortions group: molar pregnancies, abnormal fetal karyotype or infection induced abortion.



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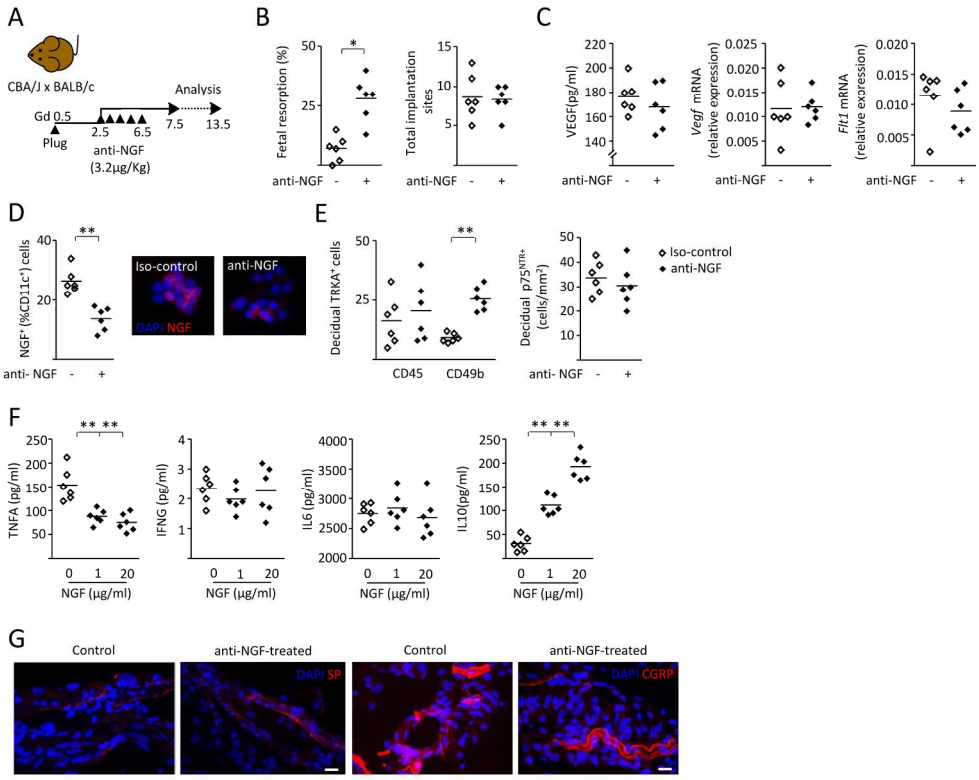

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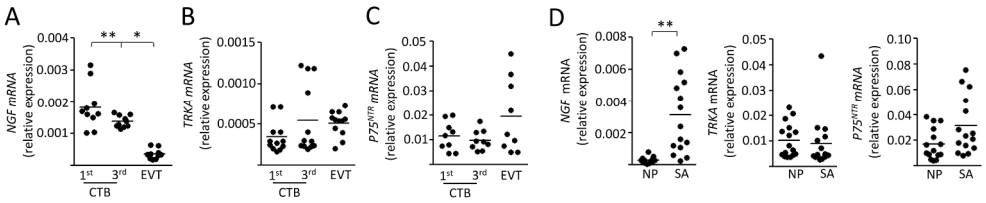

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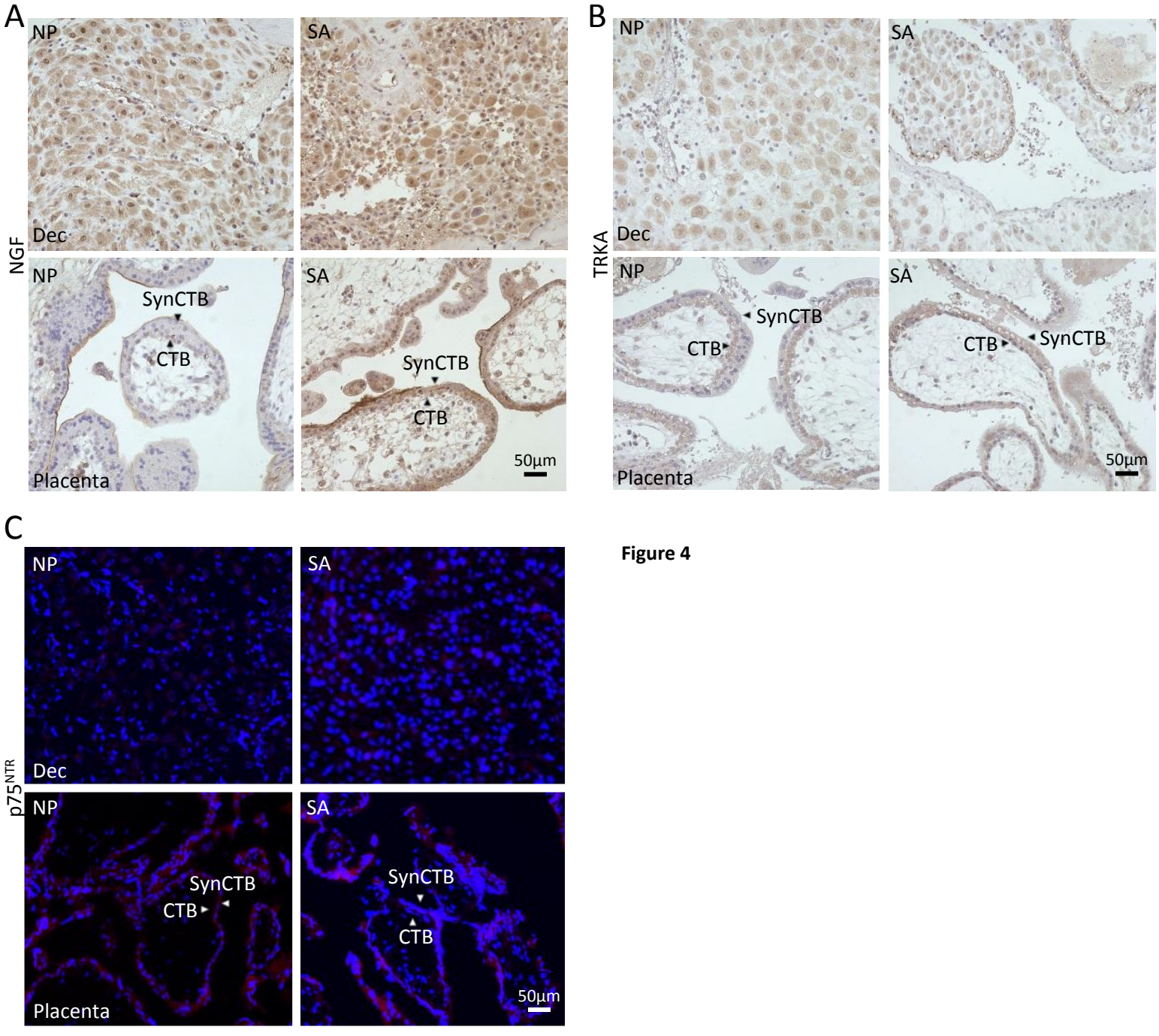
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Figure 4