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ARTICLE

Regulation of trophoblast migration and survival by a novel neural regeneration peptide

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Ambika Singh graduated with a Masters in Science (first class Honours) in 2006 from the University of Auckland, New Zealand. Her project involved studying the novel functions of a recently discovered neuroprotective peptide in human placental development during term pregnancy. She is currently in the final stages of her PhD investigating the role of sphingolipid compounds in the regulation of trophoblast differentiation, at the University of Western Australia, Australia.

Abstract Although placental trophoblast migration is tightly controlled in an autocrine/paracrine manner, the nature of chemoattractive factors facilitating and directing this biological activity remains largely elusive. Neural regeneration peptides (NRP), a recently discovered peptide family, stimulate neuronal migration, differentiation and survival of post-natal neurons within the murine central nervous system. Based on the neural-repair related activities of these peptides and parallels between neuronal and placental cell behaviour patterns, this study postulated that they play a role in placental development, in particular trophoblast migration and survival and investigated the role of a newly discovered NRP motif (NNZ-4920), which exhibits about 70% homology to the mouse NRP motif sequence and is homologous to a 13-mer fragment within the N-terminus of human *CAPS2*, in trophoblast migration and survival regulation. NNZ-4920 significantly enhanced trophoblast migration by 51% ($P < 0.01$) compared with controls and protected against stress induced by serum withdrawal and tumour necrosis factor-α/interferon-γ treatment, at femtomolar concentrations, with efficacy similar to epidermal growth factor. *CAPS2* expression was detected in purified term trophoblast and decidual cells. In conclusion, the placenta may be a source of NRP-related gene expression. Its encoded peptide products exert biological effects on term trophoblast migration and survival *in vitro*.

KEYWORDS: NRP, placenta, trophoblast migration, trophoblast survival

Introduction

During embryonic brain development, proliferative zones are formed where neuroblasts are generated. Proliferating cells subsequently migrate to their destined positions, where they differentiate into physiologically active neurons. A recently described factor, termed neural regeneration peptide (NRP), has been shown to effectively enhance murine neuroblast survival, proliferation, migration and differentiation at femtomolar concentrations (Gorba et al.,

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2006). Its effects were blocked by administration of a neutralizing antibody against the chemokine receptor CXCR4, implying it may exert its actions via this plasma membrane receptor. The novel human-derived synthetic peptide NNZ-4920 (H-REGRRAAPGRAGG-NH_2) exhibits 69.5% homology to the mouse orthologue NRP sequence (a fragment from the 135-amino-acid NRP encoded by the *Nrp* gene (GenBank NM_001013372) (Gorba et al., 2006). NNZ-4920 represents the amino acid residues 38—50 within the N-terminus of the human protein calcium-dependent activator protein for secretion (CAPS-2), which is strongly expressed in the brain and endocrine cells. *CAPS2*, one of three CAPS gene isoforms, displays differential spatiotemporal expression to that of *CAPS1* in adult and fetal tissues (Cisternas et al., 2003), indicating differential cellular function of these two molecules despite sharing more than 93% homology (Sadakata et al., 2004). The only major site of non-homologous resides is in their N-terminal regions i.e. the NNZ-4920 location. Neuronal studies have demonstrated CAPS2-mediated enhanced survival of Purkinje cells by promoting a depolarization-induced release of neurotrophins, such as neurotrophin 3 and brain-derived neurotrophic factor (Sadakata et al., 2004). Interestingly, the NNZ-4920 peptide portrays strikingly similar chemoattractive characteristics to stromal cell-derived factor 1/chemokine (C-X-C motif) ligand 12 (SDF1/CXCL12), the primary agonist of chemokine (C-X-C motif) receptor 4 (CXCR4) (Gorba et al., 2006).

The placenta expresses many peptides and receptors that are traditionally thought of as being neuronal in terms of origin and function. However, although the profile and expression may be similar, the function and regulation of these peptides often varies considerably. Similar to embryonic brain development, trophoblasts undergo continuous proliferation before migrating to their zone of maturation/differentiation (Mayhew and Barker, 2001). In addition, placental trophoblasts and neuronal cells both exhibit CXCR4 and SDF1 responsiveness. The factors and processes involved in the regulation of human placental development are complex and only partially understood. Trophoblast function is stringently regulated in an autocrine/paracrine manner by growth factors, binding proteins and matrix constituents such as proteoglycans. Disordered decidual—trophoblast interactions have been implicated in spontaneous early pregnancy miscarriages, while insufficient trophoblast invasion of spiral arteries has been closely linked with pregnancy complications such as pre-eclampsia and intrauterine growth restriction (Brosens et al., 1972; Hustin et al., 1990; Robertson et al., 1975; Smith et al., 1997).

Interestingly, *CAPS2* expression has been detected in the placenta (Cisternas et al., 2003), although the specific cell types expressing this gene in the placenta remain unknown. Based on the known activities of NRP, its localized expression within regions of elevated cell migratory activity and analogies between neuronal and placental cell morphology during migratory movement and *CAPS2* expression, this study postulated that NNZ-4920 may display chemoattractive and survival properties in trophoblast cells. To assess the ability of the human placenta to produce NRP-like peptides, expression of *CAPS2* was also investigated.

Materials and methods

The following reagents were purchased from commercial sources: DNase I and interferon γ (IFNγ) (Roche Diagnostics, Basel, Switzerland); Percoll (Amersham Biosciences, New Jersey, USA); M199 media, fungizone, trypsin-EDTA, fetal bovine serum, bovine serum albumin, laminin, phosphate-buffered saline (PBS), sodium dodecyl sulphate, fibronectin, syto-24 stain, Tris, acetic acid and EDTA buffer, dithiothreitol and penicillin/streptomycin (Invitrogen, Carlsbad, USA); GoTaq Flexi DNA polymerase, ImProm-II reverse transcription system and GoTaq Hot Start polymerase (Promega, Madison, USA); dNTP (Bioloine, Toronto, Canada); D-(+)-glucose and paraformaldehyde (Scientific Supplies, Auckland, New Zealand); tumour necrosis factor α (TNFα) (PreProTech, Canton, USA); epidermal growth factor (EGF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and poly-D-lysine (PDL; Sigma—Aldrich, Castle Hill, Australia); Cy3-labelled goat anti-mouse antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK); and anti-cytokeratin 7 antibody (DacoCytomation, Glostrup, Denmark).

Manufacture and stabilization of the peptide NNZ-4920

NNZ-4920 (H-REGRRAAPGRAGG-NH_2) was manufactured by Auspep (Parkville, Victoria, Australia) as a trifluoroacetate salt with an amidated C-terminus (theoretical molecular weight of 1285.5). The only difference to the human *CAPS2* sequence motif (amino acids 38—50) is a switch from aspartate (position 6) to alanine purely for stability purposes. D-(+)-trehalose was added to the peptide (w/w ratio 167:1) during lyophilization to prevent water uptake during storage. Prior to each experiment, NNZ-4920 was reconstituted in PBS at a concentration of 500 μmol/l. The peptide was stored at -80°C in a lyophilized state under argon pressure. Subsequent dilutions of peptides were made in PBS.

Cytotrophoblast culture

Normal delivered placentas were obtained with informed consent from women at Auckland Hospital by elective Caesarean section. Cytotrophoblasts were isolated as described earlier (Blumenstein et al., 2002). Briefly, cytotrophoblasts derived from term placenta ($n = 4$) were liberated by eight sequential digestions with 0.25% trypsin digestion, supernatants were collected in 50 ml Falcon tubes and centrifuged at 290*g* for 7 min. Erythrocytes were removed by incubation of cell pellet in a red cell lysis buffer (50 mmol/l NH_4Cl, 10 mmol/l $NaHCO_3$ and 0.1 mmol/l EDTA) and cytotrophoblasts were purified by centrifugation at 1200*g* for 20 min on a discontinuous Percoll gradient (20—60%). Cells migrating between the 40% and 50% Percoll bands were collected and plated either in 12-well transwell plates (5×10^4 cells/well) for migration assays or 96-well plates (9×10^4 cells/well) for viability studies. The cells were grown in M199 media, supplemented with 10% FCS and penicillin/streptomycin (100 U/ml) in a 5% CO_2 humidified atmosphere at 37°C. After 24 h, culture media was removed, cells were washed with PBS and media containing 1% FCS was added. Villous placental tissue was washed in saline to remove

excess maternal blood and stored for quantitative PCR. Decidua parietalis was obtained by scraping the decidua from the reflected membranes, digesting with dispase/collagenase, purification over Percoll and culturing for 3–5 days before undergoing RNA extraction.

Migration assay

To investigate the chemoattractive effects of NNZ-4920, the bottom surface of the wells was pre-coated with 0.1 ng/ml NNZ-4920 or 0.1 mg/ml bovine serum albumin as a control for 1 h at 37°C. Plates were then incubated at room temperature with 7 μg/ml laminin for 2 h, followed by a PBS wash before adding 1.5 ml of supplemented cell culture media to the wells. Inserts were coated with 10 μg/ml PDL for 15 min, washed twice with PBS, dried and placed in the wells and filled with 0.35 ml of supplemented cell culture media. NNZ-4920 (1×10^{-13} mol/l) was added to the bottom surface of the treatment wells. Trophoblasts were plated into inserts of transwell plates (12 μm pore size) and cultured for 20–24 h. Cells were then incubated for 2 h with syto-24, a live fluorescent intercalating stain. Cells that migrated to the bottom surface of the wells were then fixed with 4% paraformaldehyde, immunostained with anti-cytokeratin-7 (1:500) to confirm trophoblastic identity and quantified by counting (whole well). Immunostaining was visualized with Cy3-labelled secondary antibody on an Olympus IX 71 inverted microscope (Tokyo, Japan; data not shown).

Viability assay

Cell viability was measured spectrophotometrically as the reduction of MTT by mitochondrial succinate dehydrogenase after 48 h following various treatments (Mosmann, 1983). Cells were plated in 96-well plates in 100 μl of M199 media supplemented with FCS (1%) with/without cytokines and serum.

Real-time PCR

Total RNA was extracted from term placental tissue, cultured cytotrophoblasts and decidual cells using RNAqueous-4PCR columns (Ambion, Austin, USA). Contaminating DNA was removed during extraction by incubation with DNAse 1. The concentration and quality of RNA samples was determined by NanoDrop ND-1000 spectrophotometer. First strand synthesis was performed using the ImProm-II kit. The primer sequences for human *CAPS2* were designed using OligoPerfect Designer (Invitrogen) and sequence specificity confirmed via BLAST search. The sense (5′-TGCAGCAAGGTTATGCA GAC-3′) and antisense (5′-TCTTCTTCTCTGCAGCCACA-3′) primers amplified a 115 base-pair fragment of human *CAPS2* mRNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase served as a positive control. Samples were amplified in a 24.5 μl PCR reaction mix (5 μl 5× PCR buffer, 2 μl 2 mmol/l $MgCl_2$, 0.5 μl 10 mmol/l dUTP, 1.25 μl EvaGreen, 1 μl 400 nmol/l primers, 0.125 μl GoTaq Hot Start polymerase, 1 μl sample cDNA plus diethylpyrocarbonate-treated water to make up 24.5 μl) for 40 cycles. PCR products were subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis

To test for significance of the experimental results the non-parametric Mann–Whitney *U* and Kruskal–Wallis tests were applied, using Dunn's multiple comparison test as post-hoc test. All data are represented as mean values ± standard error.

Results

NNZ-4920 enhanced migration of primary term human trophoblasts

Primary cytotrophoblast from term placentas were employed in haptotactic migration studies. In preliminary studies, different extracellular matrix (ECM) proteins, including fibronectin, laminin, PDL and Matrigel, were analysed to determine optimal conditions for placental trophoblast migration and subsequent adherence and survival, since term trophoblasts possess only limited migratory capabilities. While Matrigel is a commonly used ECM protein for migration assay, fibronectin and laminin were selected on the basis of them being ligands to integrins, which are the major family of cell adhesion molecules important for trophoblast cell migration. They are expressed in cytotrophoblasts and allow these cells to recognize their immediate environment and adapt to it. Integrins mediate adhesion of trophoblasts to ECM proteins (Hynes, 1992; Kuhn and Eble, 1994). In this study, maximum cell migration was observed when the bottom surface of the wells was coated with 0.1 ng/ml NNZ-4920 (7.5×10^{-11} mol/l) and 7 μg/ml laminin, while the insert membrane was coated with 10 μg/ml PDL. Addition of 1×10^{-13} mol/l NNZ-4920 to the wells induced a 51 ± 17% increase (mean ± SEM; $n = 3$) in migration of term trophoblasts compared with control conditions (**Figure 1**). The experimental design and optimal NNZ-4920 concentration have been adopted from published neuronal studies (Gorba et al., 2006). This concentration of NNZ-4920 is within the maximal biological activity range detected in neuronal cell migration assays (Gorba et al., 2006). Over 97% of the cells that had migrated to the bottom surface of the wells were trophoblasts (cytokeratin 7-positive) in both NNZ-4920 and control conditions. Individual experiments were normalized to controls and pooled data were presented as a percentage of cells migrated compared with controls.

Increases in cell viability

A comparison was made between the protective effects of a commonly used survival factor, EGF and NNZ-4920 against spontaneous cell death *in vitro* (**Figure 2**). EGF was used at 0.3, 0.8 and 3×10^{-9} mol/l, while NNZ-4920 was administered at concentrations ranging from 1×10^{-15} to 10^{-11} mol/l for a duration of 48 h. NNZ-4920 enhanced cell viability by 31 ± 4% at 1×10^{-15} mol/l compared with control conditions (untreated cells); this effect was comparable in magnitude to the 29 ± 4% increase in cell viability demonstrated by

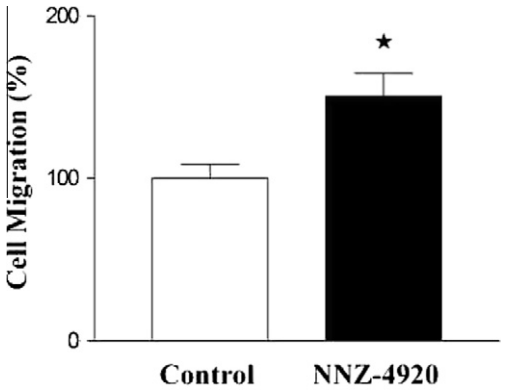

Figure 1 Haptotactic migration of trophoblasts treated with NNZ-4920. NNZ-4920 (1×10^{-13} mol/l) caused a significant increase in trophoblast migration by 51 ± 17% as compared with control (media supplemented with 0.01% bovine serum albumin). Trophoblasts were plated and allowed to migrate for 2024 h. Cells that had migrated to the bottom surface of the well were fixed with 4% paraformaldehyde, immunostained for cytokeratin-7 (trophoblast specific marker; data not shown) and counted. Data shown are a percentage of mean values ± SEM of pooled replicates normalized to controls. *$P = 0.0125$; $n = 3$; Mann–Whitney *U*-test.

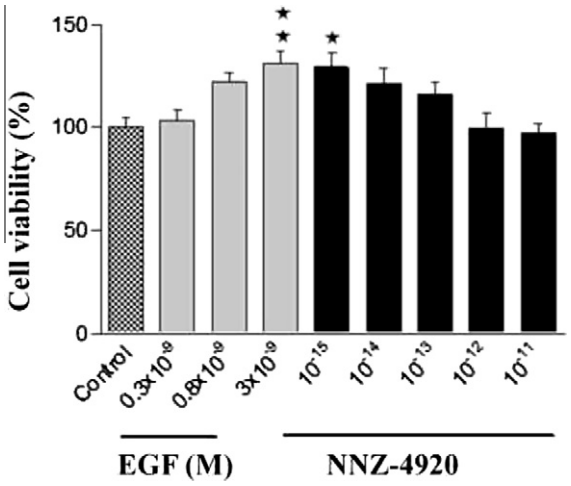

Figure 2 Effects of NNZ-4920 on spontaneous cell death *in vitro* in term placental trophoblast. Trophoblasts were treated with a range of concentrations of NNZ-4920 and EGF, in culture media supplemented with 1% serum, for 48 h. After 48 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was carried out. NNZ-4920 enhanced cell viability by 31 ± 4% at 1×10^{-15} mol/l compared with control conditions (untreated cells); this effect was comparable in magnitude to the 29 ± 4% increase in cell viability demonstrated by 3×10^{-9} mol/l EGF. Results were normalized to control (media alone supplemented in 1% serum) within each experiment. Data shown are a percentage of mean values ± SEM of pooled replicates. EGF = endothelial growth factor; *$P < 0.05$; **$P < 0.01$; $n = 3$; Kruskal–Wallis test, with Dunn's multiple comparison test as post-hoc test.

3×10^{-9} mol/l EGF. Individual experiments were normalized to controls and pooled data were presented as a percentage of number of viable cells compared with controls.

In addition, two separate injury paradigms causing significant decreases in viability were used to further investigate the protective effect of NNZ-4920, namely TNFα (100 ng/ml)/IFNγ (100 U/ml)-induced stress and serum withdrawal. Cells exposed to cytotoxic stimuli were simultaneously treated with NNZ-4920 (1×10^{-15} to 1×10^{-11} mol/l) and EGF (0.8×10^{-9} mol/l) was used as a positive control. Under serum withdrawal conditions, NNZ-4920 showed a bell-shaped dose response curve with recovery from significant physiological insult following serum withdrawal (**Figure 3**). Albeit not significant, the protective trend in response to NNZ-4920 after serum-withdrawal is similar to that of stress-inducing cytokine exposure implying comparable effects in response to both injury paradigms. EGF (0.8×10^{-9} mol/l) also protected against the cytotoxic effects of serum withdrawal, increasing viability by 15 ± 3% above control, similar to the effects observed with femtomolar concentrations of NNZ-4920. Previous studies have established that synergistic effects of TNFα and IFNγ induce trophoblasts apoptosis, which is abrogated by EGF (Smith et al., 2002). In agreement with these findings, EGF (0.8×10^{-9} mol/l) significantly protected against toxicity induced by TNFα/IFNγ, increasing MTT activity by 16 ± 2% compared with control $P < 0.05$; **Figure 4**). Administration of NNZ-4920 also resulted in significant protection from the toxic effects of TNFα/IFNγ at 1×10^{-15} and 1×10^{-14} mol/l, enhancing viability by 22 ± 3% and 21 ± 3%, respectively.

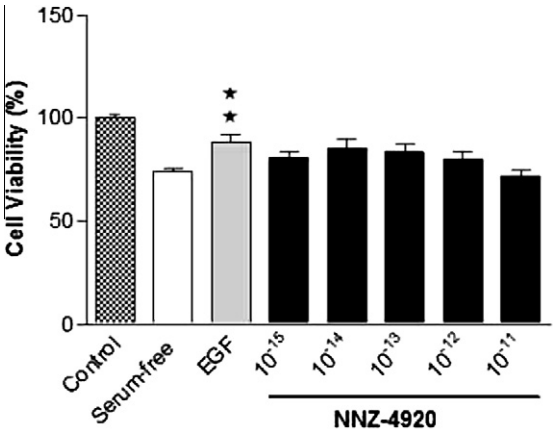

Figure 3 Protective role of NNZ-4920 against cytotoxic effects induced by serum withdrawal on trophoblasts *in vitro*. Trophoblasts were incubated in serum free media for 48 h after administration of NNZ-4920 and endothelial growth factor (EGF) (positive control); followed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. NNZ-4920 showed a bell-shaped dose response curve with recovery from significant physiological insult following serum withdrawal, albeit not significant. EGF (0.8×10^{-9} mol/l) also protected against the cytotoxic effects of serum withdrawal, increasing viability by 15 ± 3% above control, similar to the effects observed with femtomolar concentrations of NNZ-4920. Results were normalized to control (media supplemented with 10% serum) within each experiment. Data shown are a percentage of mean values ± SEM of pooled replicates. **$P < 0.01$; $n = 4$; Kruskal–Wallis test, with Dunn's multiple comparison test as post-hoc test.

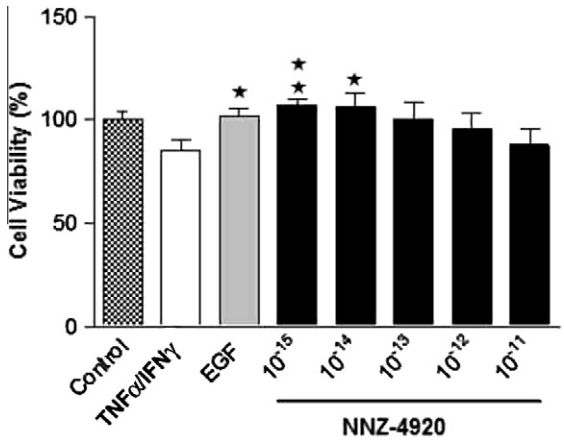

Figure 4 Effects of NNZ-4920 on cytotoxicity induced by tumour necrosis factor α (TNF α) and interferon γ (IFNγ) on trophoblasts *in vitro*. Trophoblasts were treated simultaneously with NNZ-4920, endothelial growth factor (EGF) (positive control) and cytokines (TNF α plus IFNγ) for 48 h. After 48 h incubation, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EGF (0.8×10^{-9} mol/l) significantly protected against toxicity induced by TNFα/IFNγ, increasing MTT activity by 16 ± 2% compared with control. Administration of NNZ-4920 also resulted in significant protection from the toxic effects of TNFα/IFNγ at 1×10^{-15} and 1×10^{-14} mol/l, enhancing viability by 22 ± 3% and 21 ± 3%, respectively. Results were normalized to control (media alone supplemented with 1% serum) within each experiment. Data shown are a percentage of mean values SEM of pooled replicates. $^{*}P < 0.05$; $^{**}P < 0.001$; $n = 3$; Kruskal–Wallis test, with Dunn's multiple comparison test as post-hoc test.

Although the potency of NNZ-490 is remarkable, it should be noted that the dose–response curve of NNZ-4920 is similar to that observed in neuronal studies, where most survival-promoting activity after oxidative/excitotoxic stress occurred in the femtomolar range with higher concentrations exerting no protective effect (Gorba et al., 2006). No significant toxic effects of the peptide were observed using this injury paradigm.

CAPS2 mRNA expression in term human placenta

An amplicon of the predicted size (115 bp) was detected upon quantitative PCR analysis of cDNA derived from whole placental tissue, purified trophoblast and decidual cells (**Figure 5**; $n = 4$, lanes 1–4). Interestingly, expression levels of *CAPS2* between different cells types were similar showing no significant difference. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used to confirm cDNA quantity and quality.

Discussion

Placentation in humans is characterized by tightly regulated trophoblast differentiation, proliferation, migration and subsequent invasion into the decidua and spiral arteries. Dysregulation of these trophoblast activities during placental development leads to a number of pathologies such as early pregnancy loss, pre-eclampsia and intrauterine growth restriction. The current study investigated the effects of NNZ-4920, a synthetic peptide with NRP-like properties, on trophoblast migration and survival under in-vitro conditions. This 13-amino-acid peptide, which is homologous to a fragment of the N-terminal region of human CAPS2 protein, has been shown to effectively enhance neuronal survival, proliferation, migration and differentiation (Gorba et al., 2006). The data presented here suggest that its biological effects in the placenta may be similar to those observed in the central nervous system.

Trophoblast migration is a two-wave phenomenon, involving the migration of cells down the cell column toward the maternal decidua and subsequent trophoblast invasion into the decidua and spiral arteries (Pijnenborg, 2000). Although trophoblast migration has been reported to be restricted primarily to the first trimester of pregnancy (Pijnenborg et al., 1980), placental tissue from first-trimester pregnancies was not available for this study. The present study, therefore, developed a model to explore trophoblast migration induction in term placental tissue. To retain cytotrophoblasts in their mononuclear phenotype and prevent syncytialization, reduced serum culture medium was used. Interestingly, trophoblast migration was detected under control conditions in this study using purified term cytotrophoblasts. Previous research has shown that villous cytotrophoblasts are stem cells and can spontaneously transform once released from their normal villous surroundings into an intermediate extravillous cell type possessing migratory characteristics (Pijnenborg, 1994). Furthermore, studies have cited similarities between characteristics of extravillous trophoblasts and cultured villous cytotrophoblasts (Pijnenborg et al., 1996).

In agreement with findings from neuronal cells, the current study supports a possible role for NRP in the regulation of trophoblast migration during pregnancy. For in-vitro migration studies, the choice of ECM is a critical factor to be considered when simulating in-vivo conditions, as cellular factors do not solely control cell motility; the ECM also plays a crucial role (Burrows et al., 1996). In this study, maximum cell migration was demonstrated when the bottom surface of the wells was coated with NNZ-4920 and laminin, with PDL used to coat insert membranes. The removal of PDL from these assays abolished any migratory activity. While PDL (a positively charged molecule) serves as an attractant for negatively charged proteins, laminin functions as an adhesion molecule by specifically binding to the laminin receptor located on the plasma membrane of trophoblasts after cells have migrated to the bottom of the wells. It is speculated that the ligand (NNZ-4920), liberated from the plate, binds to a yet-to-be unidentified chemokine receptor and directs migration of trophoblasts towards the NNZ-4920 source. Neuronal studies demonstrated an inhibition in mouse NRP-mediated cell migration by anti-CXCR4 neutralizing antibody, implying this being its mode of action (Gorba et al., 2006). Expression of chemokine receptor CXCR4 and its predominant ligand SDF1/CXCL12, in migrating/invading cytotrophoblasts and decidual cells has been shown to enhance their coordination and communication, resulting in an improved functional

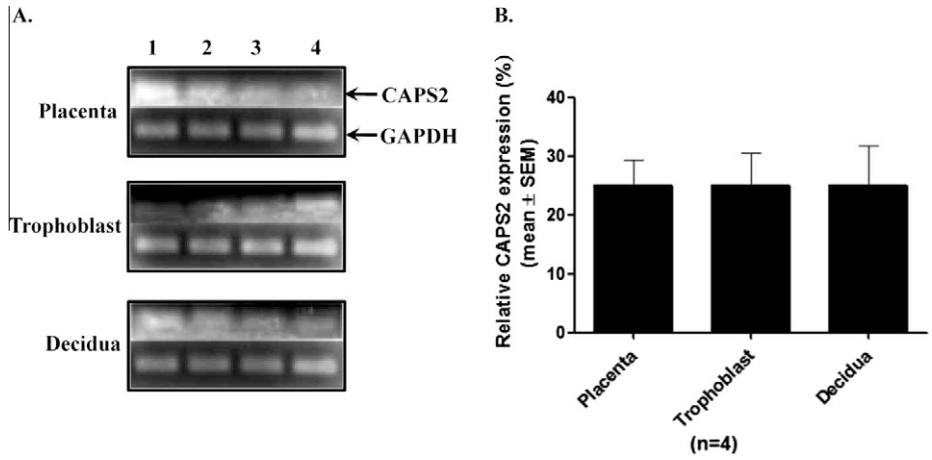

Figure 5 *CAPS2* mRNA expression in the term trophoblast cells and decidual cells. Quantitative PCR was carried out on cRNA from term human placenta and purified trophoblast and decidual cells using *CAPS2*-specific primers. (**A**) Expression of *CAPS2* was detected in term placental tissue, purified cytotrophoblasts and decidual cells and (**B**) with levels of expression being similar between different sources. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used to confirm cDNA quantity and quality.

maternal–fetal interface (Hanna et al., 2003; Mayhew et al., 1999; Yang et al., 2006; Zhou et al., 2008). In addition to cytotrophoblasts expressing SDF1, these cells were also found to secrete the chemokine, which chemoattracts decidual lymphocytes expressing CXCR4, thereby modulating the immune milieu at the maternal–fetal interface (Wu et al., 2005; Yang et al., 2006). It is possible that NNZ-4920 mediates its migratory effects by binding to CXCR4, similar to SDF1, although this has yet to be confirmed. Endogenous SDF1 may contribute towards the basal levels of trophoblast migration observed under control conditions; however, in the current study endogenous concentrations of SDF1 in the media are unknown. More recently, studies have identified the expression of a number of other chemokines and their receptors, such as chemokine (C-C motif) ligands 4 and 14 (CCL4 and CCL14) and chemokine (C-X3-C motif) ligand 1 (CX3CL1), at the maternal–fetal interface and reported their involvement in targeted trophoblast migration *in vitro* (Caballero-Campo et al., 2002; Hannan et al., 2006; Jones et al., 2004; Red-Horse et al., 2001). Whether the increase in trophoblast migration in response to NNZ-4920 is a result of direct potential receptor activation or indirectly via activation and/or up-regulation of other trophoblast migration enhancing factors, is yet to be determined. Interestingly, the current study detected expression of *CAPS2* in purified cytotrophoblasts and decidual cells, suggesting that a *CAPS2*-derived NRP-like peptide derived from the decidua may help direct trophoblast migration towards the decidua via its chemoattractive functions *in vivo*. The mechanisms underlying trophoblasts homing to the maternal vasculature are not well understood and, thus, the likelihood that NRP-like peptides contribute to the directional migration and homing of trophoblasts to the endometrium is important. Further analysis of the signals transduced via the ECM microenvironment and the coordinated effect of various growth factors and cytokines will provide a better understanding of the potential contribution of NRP-like peptides on the migratory behaviour of trophoblasts *in vivo*. This may lead to the development of regimes to treat pregnancy disorders associated with inadequate placentation.

Trophoblast viability is regulated by multiple factors, which activate and/or suppress the apoptotic cascade in a spatiotemporal manner during human placental development (Mayhew et al., 1999; Smith et al., 1997). Apoptosis is a normal feature of trophoblast lifecycle *in vivo* and *in vitro* and increases as gestation progresses. However, excessive apoptosis diminishes placental function, as shown in serious complications of pregnancy such as pre-eclampsia and intrauterine growth restriction. NNZ-4920 inhibited the decline in cell viability induced by serum withdrawal (i.e. removal of mitogenic and anti-apoptotic growth factors). The magnitude of this protective effect of NNZ-4920 after serum withdrawal was similar to that of stress-inducing cytokine paradigm, suggesting that it was likely to be a real phenomenon. In addition, EGF, which has previously been shown to exert no protection against serum withdrawal (Desai et al., 1999), also rescued trophoblasts from stress-induced cytotoxicity in this study. NNZ-4920 also significantly protected against TNFα/IFNγ-induced toxicity in cultured term cytotrophoblasts. Synergistic effects of TNFα/IFNγ in inducing cytotoxicity have been widely reported, as has inhibition of these cytotoxic effects by EGF (Garcia-Lloret et al., 1996; Smith et al., 2002; Yui et al., 1994). In agreement with previous findings, EGF showed significant protection against the toxic effects of TNFα/IFNγ in primary trophoblasts in this study. EGF's inhibitory actions on cytotoxic effects may be related to its ability to up-regulate syncytialization and/or to stimulate trophoblast survival (Garcia-Lloret et al., 1996). Smith et al. showed that cytotrophoblasts are less susceptible to cytokine-induced apoptosis when they syncytialize, possibly due to a loss of TNFα receptors or decline in fragility as cytotrophoblasts fuse to form a syncytium (Smith et al., 2002). SDF1 has also been shown to mediate trophoblast survival by inducing differentiation (Jaleel et al., 2004). Although NNZ-4920 does display pro-survival characteristics in neuronal and placental cells, it is yet to be determined

whether it does so by directly stimulating trophoblast viability or by acting as a pro-differentiation compound. However, the latter seems unlikely since a gradual decline in cell viability is observed as trophoblasts undergo differentiation *in vitro* (Singh et al., unpublished data). Therefore, it is unlikely the enhanced viability seen in response to NNZ-4920 is due to an up-regulation in syncytial formation.

Unlike several cytoprotective factors, NNZ-4920 displays potent *in-vitro* biological activity in the femtomolar dose range. In addition, this peptide exhibits a bell-shaped dose—response as seen in the present study, showing a protective trend at lower concentrations and non-effective in the nanomolar range. This type of dose—response curve is typically exhibited by neurotrophins such as brain-derived neurotrophic factor and neurotrophin 3, which regulate neuronal cell survival and differentiation within the CNS (Sadakata et al., 2004). They exert their trophic effects in response to CAPS2-mediated neurotrophin secretion following membrane depolarization.

Cisternas et al. (2003) previously showed that *CAPS2* is expressed in the human placenta, but its cellular origin remained unknown. The present study not only confirmed these initial findings, but further demonstrated that *CAPS2* is also expressed in purified cytotrophoblasts and decidual cells. The placenta provides an important physiological, as well as a metabolic, barrier protecting the fetus from trauma, drugs and endogenous toxins derived from the maternal circulation. Given that expression of *CAPS2* was detected in the trophoblast and decidual cells, and that NNZ-4920 protects against cytotoxic insult by cytokines in trophoblast cells, it is possible that a *CAPS2*-derived peptide may play a similar protective role *in vivo*. Furthermore, a protective role against infections such as HIV transmission, as shown for SDF1, by preventing entry of CXCR4-specific HIV strains may potentially be another function of these peptides (Coulomb-L'Hermine et al., 2000; Earl et al., 1990).

In conclusion, this study reports the effects of a novel NRP-like molecule (NNZ-4920) in up-regulating cytotrophoblast migratory and survival properties when administered to in-vitro cultivated cytotrophoblasts. Whilst the limitations of term villous trophoblasts as a cell migration model for this study have been appreciated, further work will be needed to investigate the effects of this peptide during early gestation. NNZ-4920 also potentially plays a survival role in the femtomolar dose range, similar in magnitude to EGF. It is of interest to note that expression of *CAPS2* was detected in term placental tissue, purified cytotrophoblasts and decidual cells, with levels of expression being quite similar. The physiological significance of NNZ-4920 and the receptor/signal transduction pathway responsible for its actions await further clarification. In spite of research on this peptide still being in its infancy, the present findings are indicative of NRP motifs as attractive candidates for therapeutic applications in pregnancy disorders.

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