Molecular Human Reproduction, Vol.17, No.4 pp. 227–232, 2011
Advanced Access publication on December 22, 2010 doi:10.1093/molehr/gaq101

MHR Basic science of reproductive medicine
ORIGINAL RESEARCH

Hepcidin and iron species distribution inside the first-trimester human gestational sac

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Submitted on October 10, 2010; resubmitted on December 4, 2010; accepted on December 14, 2010

ABSTRACT: We have investigated factors affecting iron distribution in the first-trimester gestational sac, by the measurement of transferrin, non-transferrin-bound iron (NTBI) and pro-hepcidin (Hep) in maternal serum, coelomic fluid (CF) and amniotic fluid (AF) and by immunostaining for Hep in villous and secondary yolk sac biopsies. These samples were obtained from 15 first-trimester pregnancies at 8–11 weeks gestation. Transferrin concentrations were significantly lower in fetal (0.56 mg/ml) than maternal serum (1.71 mg/ml), with very low concentrations in CF and AF (0.09 mg/ml). In contrast, transferrin saturations were significantly higher in fetal (77%) than maternal serum (33%). NTBI was present in fetal serum, CF and AF, presumably as a consequence of low transferrin concentrations in these compartments. Pro-Hep was present at lower levels in fetal (140.0 ± 11.1) than maternal serum (206.2 ± 9.2) and at low concentrations in CF (19.4 ± 3.1) and AF (21.8 ± 5.2). Immunostaining with Hep antibody was found in the syncytiotrophoblast of first-trimester placenta as well as in mesothelial and endodermal layers of the secondary yolk sac at 10 weeks. The presence of Hep in syncytiotrophoblast cells of first-trimester placenta as well as in mesothelial and endodermal layers of the secondary yolk sac suggest a key regulatory role for this protein in iron transfer to the first-trimester fetus. The low transferrin concentrations and the presence of NTBI in CF and AF suggest that transferrin-independent iron transfer is important in early gestation.

Key words: amniotic fluid / coelomic fluid / hepcidin / yolk sac / placenta

Introduction

Human iron homeostasis results from tightly coordinated regulation by the various proteins involved in iron uptake, transport, intracellular storage and trafficking of iron (Rhoades and Pflanzer, 1992). The human fetus and placenta accumulate around 350 mg of iron during pregnancy. In adults, two-thirds of the 3000–4000-mg total body iron content is found in haemoglobin. Most of the remaining iron is contained in myoglobin and respiratory proteins, stored bound to ferritin in the liver and spleen, or combined with transferrin. Transferrin transports the iron absorbed by the intestinal wall or released from hepatic stores through the plasma for delivery to the bone marrow and other iron-requiring tissues. In primates, including humans, the greatest source of iron transferable to the fetus is that bound to maternal transferrin, and the amount of iron needed by the fetal tissues is an obligatory requirement that is met regardless of maternal iron stores. The rate of iron transfer across the placenta increases with fetal growth and has been related to the development of fetal haematopoiesis (Rhoades and Pflanzer, 1992; McArdle *et al.*, 2003).

Maternal iron is released into the intervillous space and is rapidly taken up by transferrin receptors located on the surface of the syncytiotrophoblast (Seligman *et al.*, 1979; Wada *et al.*, 1979). Cellular iron is probably transported in an uncharacterized low-molecular-weight form in iron storage granules in the syncytiotrophoblast (Okuyama *et al.*, 1985; Knisely *et al.*, 1989) or stored bound to ferritin (Brown *et al.*, 1979) and is released into the fetal circulation to be bound to transferrin. The anatomy of the first-trimester gestational sac in humans is different to that of other mammalian species and to that of the second and third trimester (Jauniaux and Gulbis, 2000). In the first-trimester pregnancy, the secondary yolk sac floats within the exocoelomic cavity (ECC), is directly connected to the fetal gut and possesses a rich vascular plexus in contrast with that of early placental villi. The ECC, which lies between the primitive placenta and the amniotic cavity, is the largest anatomical space inside the gestational sac between 5 and 9 weeks of gestation. At the end of the

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first trimester, the secondary yolk sac starts to degenerate, the ECC is progressively obliterated by the growing amniotic cavity and the fetal circulation shifts from a mainly vitelline to an exclusively villous circulation. The study of the biochemical composition of the coelomic fluid (CF) in parallel with that of the amniotic fluid (AF), and the anatomy and biological activities of the surrounding tissue has enabled us to better understand materno-fetal transfer and to demonstrate the presence of different metabolic pathways in the first trimester of pregnancy compared with later in pregnancy (Jauniaux et al., 1991, 1996, 2003, 2004, 2007; Gulbis et al., 1998).

As iron is an obligate requirement for feto-placental development and biological activities, it is important to elucidate the mechanisms by which fetal iron is acquired and distributed within the first-trimester gestational sac. We have previously found that the concentration of iron in the CF increases between 7 and 12 weeks of pregnancy, indicating that placental iron transfer increases with advancing gestation (Gulbis et al., 1994). We have also shown that the ECC contains very high levels of ferritin when compared with maternal serum and AF, suggesting that this embryonic cavity is the main reservoir for iron in early pregnancy. The distribution of iron and other iron-binding proteins between the maternal and feto-placental compartments in the first trimester is comparable with that found later in gestation, suggesting that placental transfer of iron, other oligo-elements and vitamins may occur as early as the formation of tertiary villi (Gulbis et al., 1994; Jauniaux et al., 2004, 2007).

Ferritin serves to store iron in a non-toxic form and transport it to various tissues. However, little is known about the release of iron from ferritin *in vivo*. Complete proteolytic destruction of the molecule may be required to secure iron retrieval (Radisky and Kaplan, 1998) This suggests that ferritin in the ECC may not provide a direct delivery route for iron to the developing fetus. We have therefore investigated the presence of other iron-carrying molecules in both the fetal fluids and blood. Plasma transferrin is the iron transporter in humans and when transferrin becomes saturated, non-transferrin-bound iron (NTBI) appears in the plasma and can be taken up into cells (Evans et al., 2008). Hepcidin (Hep) is a recently described amphipathic β-sheet hairpin peptide, which is expressed mainly in the liver, as a longer precursor known as Pro-Hep (Ganz, 2004; Hugman, 2006; Zhang and Enns, 2009) This iron-regulatory hormone inhibits iron absorption in the small intestine and is regulated by hypoxia, anaemia, inflammation, dietary iron and iron stores (Ganz and Nemeth, 2010). It also possesses antimicrobial properties. Although it has been suggested that, *in utero*, Hep inhibits iron transport from maternal blood across the placenta to the fetal circulation, little is known about the distribution of this molecule inside the first-trimester gestational sac. The aim of this study was therefore to evaluate the distribution of Hep molecules in early fetal fluids, tissues and blood and its possible role in the distribution of iron-carrying molecules inside the first-trimester gestational sac.

Materials and Methods

Subjects and samples

CF, AF, fetal blood and maternal blood were obtained at 8–11 weeks of gestation prior to elective surgical termination of pregnancy under general anaesthesia. Gestational age was determined from the first day of the last menstrual period and confirmed by ultrasound measurement of the fetal crown-rump length. Written consent was obtained from each woman after receiving complete information on the procedure. The study included only uncomplicated pregnancies and was approved by University College London Hospitals Committee on the Ethics of Human Research.

CF ($n = 10$), AF ($n = 6$) and intra-cardiac fetal blood ($n = 5$) samples were obtained by transvaginal puncture under sonographic guidance as described previously (Jauniaux et al., 1991, 1996, 2007; Gulbis et al., 1998). AF and fetal blood samples could not be obtained before 8 and 10 weeks of gestation, respectively. The first 0.2 ml of CF and AF was discarded to decrease the risk of contamination by maternal blood. In all cases, maternal venous blood ($n = 15$) was obtained from an antecubital vein during the surgical procedure, and placental and secondary yolk sac tissues were collected at the end of the procedure. Samples of maternal and fetal serum and of fetal fluids were stored at -80°C until assayed.

Three samples of placental villi and decidual tissue and three intact secondary yolk sacs at 9, 10 and 11 weeks of gestational age were fixed for immunohistochemistry in 4% paraformaldehyde and embedded in paraffin wax at the end of the surgical procedure.

Bioassays

Samples were assayed for Pro-Hep, transferrin concentration and saturation and NTBI. Due to the lack of availability of commercial methods for measuring Hep, the concentration of Pro-Hep, the pro-hormone of Hep was measured in the different series of samples. Pro-Hep ELISA kits were obtained from DRG International Ltd (Boldon, Tyne and Wear, UK) and used according to the manufacturer's instructions. The detection limit for this assay was <3.95 ng/ml and the inter-assay coefficient of variations were 9.76, 6.68 and 4.82% for low-, medium- and high-range samples.

Transferrin concentration was measured using a commercially available human transferrin ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA). The minimum detection limit for the assay was 50 ng/ml and the inter-assay coefficient of variation was 7.0 and 5.5% for low- and high-range samples, respectively.

The urea gel method of Evans and Williams (1980) was used to determine transferrin saturation. In brief, polyacrylamide gels are formed in the presence of 6 M urea which partially denatures the transferrin molecule to an extent determined by the degree of iron saturation. Apotransferrin, with no bound iron, denatures the most, followed by the C- and N-terminal monoferric forms with completely saturated diferric transferrin denaturing the least leaving the diferric form as a more compact globular species able to migrate more freely through the gel matrix. Apotransferrin is the least globular and the four forms are separated when a potential difference is applied to the gel. The bands were stained using Coomassie Blue and quantitated by scanning the gel into an imaging Programme (Scion Corporation, Frederick, MD, USA).

NTBI quantification was evaluated by the method described by Singh et al. (1990). In brief, a high concentration of a relatively non-specific ligand, nitrilotriacetate (NTA) is used to bind the NTBI, which is then transferred to a specific hydroxypyridinone ligand to form a coloured iron complex that can be quantitated by HPLC. As it is difficult to exclude contaminant iron from NTA, the assay was run using NTA containing a standard added concentration of iron. Under the conditions used for the NTBI assay, no iron is removed from transferrin by NTA. Lower detection limit for the assay was a concentration of 0.5 μM. The inter-assay coefficient of variation for this assay in our hands was 17.3% measured over 29 samples and 1 year of sampling.

Immunohistochemistry

Paraformaldehyde-fixed placental villi and secondary yolk sac tissues embedded in paraffin wax were sectioned at 7 μm. After dewaxing and blocking of endogenous peroxidases by incubation with 3% H_2O_2 for 30 min, the sections were incubated with non-immune serum for 20 min. Hep antibody (Abcam, UK; used at 1/500) was applied overnight at 4°C, and binding was detected using Vectastain Elite ABC kits (Vector Laboratories) and SigmaFast DAB (Sigma), according to the manufacturers' instructions. Sections were lightly counterstained with haematoxylin.

Statistical analysis

A biomedical data processing statistical package (Statgraphics, Manugistics, Rockville, MD, USA) was used for the analysis. Data were normally distributed and are presented as the mean and standard error of the means (SEM). Differences in means of fetal fluids and maternal values were tested using the analysis of variance and the *t*-test. Results were considered statistically significant at $P < 0.05$.

Results

Immunohistochemical staining

Immunolabelling for Hep and cytokeratin 7 was used to identify Hep-positive cells in the first-trimester villi, decidua and secondary yolk sac. In the placental villi, the antibody labelled the syncytiotrophoblastic covering of the villi and fetal red blood cells, whereas the cytotrophoblast, stroma and fetal capillary walls showed no staining (Fig. 1). The decidual gland cells were negative for Hep.

Hep staining was also apparent in the secondary yolk sac. The antibody labelled strongly the cytoplasm of the external mesothelial layer and to much lesser extent the internal endodermal layer (Fig. 2). The cytoplasm and nuclei of the mesothelial layer stained uniformly, whereas the endodermal cytoplasm largely did not stain and none of the endodermal nuclei reacted positively. The mesenchyme, blood islands and ducts did not show labelling. Negative control sections, which were not exposed to primary antibodies, showed no staining (not shown).

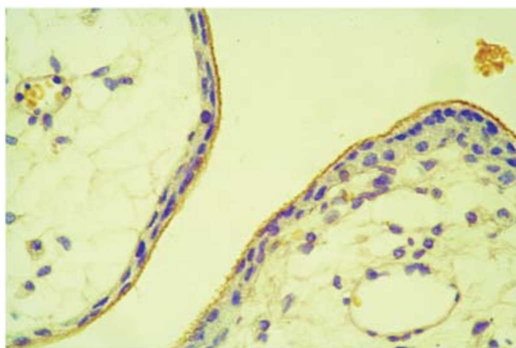

Figure 1 Immunostaining with Hep antibody (brown) in the syncytiotrophoblast cells of first-trimester placenta at 11 week and in the fetal and maternal red cells. Nuclei are stained blue (×40).

Biochemistry

Table 1 shows the concentration of transferrin and the proportion of transferrin saturated by iron (% transferrin sat) measured in the four fluid compartments. The highest levels of transferrin were found in maternal serum, where transferrin concentration in all samples was within normal range (3.0–5.2 mg/ml). Maternal serum mean transferrin concentration was significantly higher than fetal serum mean concentration ($P < 0.05$) and fluid mean concentrations (CF and AF, both $P < 0.001$). There was no significant difference in the mean transferrin concentration between the CF and the AF. Transferrin iron saturation also differed across the four fluid compartments. In maternal serum, the mean saturation was 33.1% (normal range 16–50%). Transferrin saturation in fetal serum was significantly ($P < 0.001$) higher with all individual values well above the upper limit of the range for normal adults. Transferrin saturation was difficult to measure in CF and AF samples due to the low concentrations of transferrin present. Feint diferric transferrin bands were visible on the gels containing the CF samples, whereas no bands corresponding to the other iron-containing forms were seen. There were no visible bands on gels containing the AF samples.

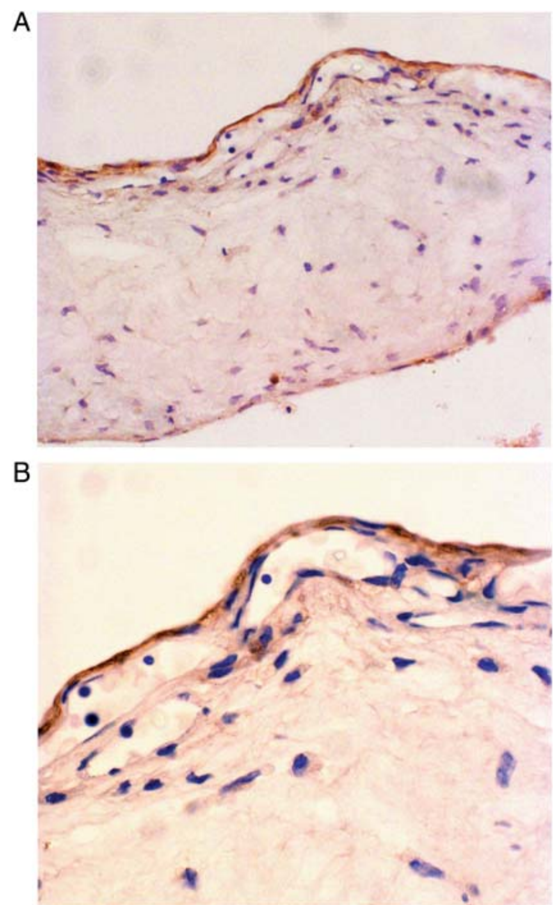

Figure 2 Immunostaining with Hep antibody (brown) in mesothelial (**A** and **B**) and endodermal (A) layers of the secondary yolk sac at 10 week. Nuclei are stained blue (A ×25; B ×75).

Table I also presents the NTBI results. There was no NTBI present in the maternal sera which had a mean NTBI value of −0.69 μM. An explanation of the negative value here will be found in the discussion. NTBI was found in the other compartments with significantly ($P < 0.01$) higher concentrations in CF than in AF. Fetal serum had the highest NTBI concentration of all fluids tested.

The Pro-Hep normal range for healthy adult humans determined by us is 73.4–163.6 ng/ml. Table II presents the mean levels of Pro-Hep measured in maternal and fetal serum and fetal fluids. Pro-Hep was not detectable in one sample of CF and in one sample of AF, and these samples have been excluded from Table II. The mean level of Pro-Hep was significantly lower in CF and fetal serum ($P < 0.001$ for both) than in MS. Pro-Hep levels in the CF and AF were similar.

Discussion

Iron absorption is tightly regulated as excessive accumulation of iron in tissues causes widespread organ damage (Halliwell *et al.*, 1992). Free iron toxicity is linked to the ability of iron to catalyse the generation of free radicals and related damaging species through the Fenton reaction, leading to oxidative stress (Halliwell and Gutteridge, 2007). The developing fetus is extremely vulnerable to oxidative stress and thus iron homeostasis must be more tightly regulated in fetal than in maternal organs and tissues (Burton *et al.*, 2003). The results of the present study confirm that the exo-CF is the main reservoir for iron in early pregnancy and that the secondary yolk sac is the principal route of entry of iron to the first-trimester fetus. Our staining data also indicate that Hep may play a role in the absorption of iron by the early fetal adnexae and its systemic distribution before the placental circulation is fully established.

Table I Comparison of the mean (SEM) values for concentrations of iron-containing species in maternal serum, CF and AF and fetal serum

Compartments	Transferrin (mg/ml)	Transferrin sat (%)	NTBI (μM)
Maternal serum ($n = 15$)	1.77 (0.19)	33.1 (1.6)	−0.69 (0.25)
CF ($n = 10$)	0.09 (0.06)	ND	3.06 (0.29)
AF ($n = 6$)	0.06 (0.03)	ND	1.58 (0.29)
Fetal serum ($n = 5$)	0.56 (0.05)	77.1 (4.5)	3.67 (0.37)

Table II Comparison of the mean (SEM) values of Pro-Hep concentration in maternal plasma, CF and AF and fetal plasma

Compartments	Pro-Hep (ng/ml)
Maternal serum ($n = 15$)	206.2 (9.2)
CF ($n = 9$)	19.4 (3.1)[a]
AF ($n = 5$)	21.8 (5.2)
Fetal serum ($n = 5$)	140.0 (11.1)[b]

[a]Significant ($P < 0.001$) difference between maternal serum and CF.
[b]Significant ($P < 0.005$) difference between maternal and fetal serum.

There is no anatomical barrier between the mesenchyme of the fetal plate of the placenta and the ECC, and protein electrophoresis has shown that the CF composition results from an ultrafiltrate of maternal serum with the addition of specific placental and secondary yolk sac bioproducts (Jauniaux and Gulbis, 2000). The higher concentrations of human chorionic gonadotrophin, estradiol, estriol and progesterone in the CF compared with maternal serum strongly suggest the presence of a direct pathway between the trophoblast and the ECC. Morphologically, this may be via the villous stromal channels and the loose mesenchymal tissue of the chorionic plate. These findings indicate that the ECC is a physiological liquid extension of the early placenta, and an important interface in fetal nutritional pathways. We have previously found that the CF contains lower iron and transferrin levels, and a higher level of ferritin compared with maternal serum, and that iron concentrations in CF increase with advancing gestation (Gulbis *et al.*, 1994). We also found no correlation between CF and maternal serum for iron and iron-binding protein levels, suggesting that the placental transfer of iron is tightly regulated and that the fetus controls its own synthesis of iron-binding proteins. In particular, the very high CF levels of ferritin found in our previous study indicate that this molecule is probably of placental and secondary yolk sac origin.

In humans, the synthesis of transferrin has been demonstrated in the fetal liver as early as 29 days of gestation (Gitlin and Biasucci, 1969) and a high level of transferrin has been measured in human fetal plasma as early as 13–14 weeks of gestation (Fryer *et al.*, 1993). In addition, transferrin molecules may traverse the placenta in very small amounts and very slowly (Gitlin *et al.*, 1964). In the present study, we found that transferrin was present at normal concentrations and that iron saturation levels were within normal ranges in the first-trimester maternal plasma (Table I). Transferrin concentrations were significantly lower in fetal serum in comparison with maternal serum, whereas the iron saturation level was much higher in fetal serum. Lower transferrin concentrations in fetal serum indicate that less transferrin iron-binding sites are available to absorb iron and this can explain the presence of NTBI in fetal serum and fluids.

NTBI is known to be a heterogeneous collection of iron-containing species, collectively measured by adding a 'gathering ligand' such as NTA (Evans *et al.*, 2008). The NTA–Fe complex is able to transfer its iron to any unsaturated transferrin present in sera resulting in negative NTBI concentrations for serum samples with low transferrin saturation such as normal sera and the maternal sera studied here (Gosriwatana *et al.*, 1999). NTBI is never present at transferrin saturations <50% and thus a mean transferrin saturation of 33.1% (Table I) excludes the presence of any NTBI in maternal serum. This situation can change later in pregnancy in cases where preeclampsia develops (Casanueva and Viteri, 2003). The very low levels of transferrin present in CF and AF precludes the absorption of NTBI iron onto transferrin. Moreover, the presence of feint diferric transferrin bands strongly suggests that this is the predominant transferrin species present in CF indicating that the transferrin is highly iron-saturated and therefore lacking in available binding sites for iron. Therefore, iron exiting from the trophoblastic cells is likely to accumulate as NTBI rather than becoming bound to transferrin in fetal fluids.

These mechanisms may be the underlying reason why NTBI is found in CF and AF.

The results of the present study show for the first time that Hep molecules are present inside the fluids and tissues of the first-trimester human gestational sac. Hep not only controls the rate of iron absorption but also determines iron mobilization from stores through negatively modulating the function of ferroportin, the only cellular iron exporter identified to date (Nemeth *et al.*, 2004). Hep molecules are pivotal for the developing fetus as high levels of Hep would inhibit iron release from placental cells. In our fluid studies, we measured Pro-Hep, an 84 amino acid peptide synthesized by the liver and from which Hep is excised through proteolytic cleavage (Hugman, 2006). Like Hep, the synthesis of Pro-Hep is enhanced by inflammation and reduced during hypoxia. In adults, Pro-Hep correlates with indices of iron status and lower levels of Pro-Hep are found in iron deficiency anaemia (Ganz, 2004). We found that the maternal serum levels of Pro-Hep in the first trimester of pregnancy were within the normal adult range (Table II). Lower levels of maternal Pro-Hep found later in pregnancy are probably associated with increased iron demand from the fetus. In the present study, the lower levels of Pro-Hep in first-trimester fetal serum samples suggest higher levels of iron absorption by the fetus. A recent study at term has shown that maternal and cord blood Pro-Hep levels correlated weakly but significantly with placental weight and relative placental size (Ervasti *et al.*, 2009). Our finding of lower concentrations of Pro-Hep in CF and AF than in maternal serum suggests a positive impact on iron mobilization from cellular stores probably via ferroportin.

Our immunohistochemistry data indicate positive staining for Hep in the villous syncytiotrophoblast and the mesothelial layer of the secondary yolk sac. The endodermal layer of the yolk sac displayed much less intense staining, whereas the decidual glands did not react at all with the Hep antibody. Using a similar approach, we have previously shown a positive immunostaining for α-tocopherol transfer protein (Jauniaux *et al.*, 2004) and folate receptors-α (Jauniaux *et al.*, 2007) in the syncytiotrophoblast, uterine glandular epithelial cells and mesothelial layer of the secondary yolk sac. These data indicate that the maternal uterine glands and the secondary yolk sac play key roles in supplying these molecules to the developing fetus before the placental circulation is established. In contrast, the data of the present study suggest that Hep in fetal fluids is produced by the secondary yolk sac. The endodermal layer of the secondary yolk sac is known to synthesize several serum proteins in common with the fetal liver, such as α-fetoprotein, albumin, pre-albumin and transferrin (Gitlin and Biasucci, 1969). With rare exceptions, the synthesis of most of these proteins is confined to the embryonic compartments, and the contribution of the yolk sac to the maternal protein pool is limited (Jauniaux and Gulbis, 2000).

The pattern of distribution of iron species in the present study is consistent with low hepcidin concentrations in developing fetal tissues and fluids which should favour iron uptake into the fetal compartments and iron egress from cells to supply the tissues of the developing fetus. In the presence of relatively low levels of transferrin, NTBI would also be expected to appear in the fluids. The question arises as to the mechanistic role of substances like hepcidin, NTBI and transferrin in supplying and transporting iron to the fetus. Whatever the mechanism, iron homeostasis has to be carefully controlled inside the early gestational sac as any imbalance may result in irreversible damage to fetal tissues (Weinberg, 2010).

Authors' roles

P.E., E.J., S.M. and J.P. designed the experiments. E.J. and S.M. collected the samples and analysed the data. P.E. and J.P. performed the bioassays and T.C-D. and G.J.B. performed the immunostaining. E.J. wrote the first draft of the manuscript which was reviewed by all authors. The final draft was approved by all authors.

Funding

Supported by a Grant from the Wellcome Trust. Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

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