Distinct Functional Roles for the Menkes and Wilson Copper Translocating P-type ATPases in Human Placental Cells

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Key Words

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Abstract

Background/Aims: The copper transporting ATPases, Menkes (ATP7A; MNK) and Wilson (ATP7B; WND) are essential for normal copper transport in the human body. The placenta is the key organ in copper supply to the fetus during pregnancy and it is one of the few organs in the body to express both of the ATPases. The placenta therefore provides a unique opportunity to elucidate the specific roles of these transporters within the one cell type. **Methods/Results:** Using polarized placental Jeg-3 cells, siRNA technology and radio-labelled ⁶⁴Cu transport assays, MNK and WND were shown to have distinct roles in the vectorial transport of copper. MNK transported copper from the cell via the basolateral membrane and in contrast, WND transported copper from the apical membrane. Inactivation of MNK resulted in decreased activity of two important cuproenzymes, lysyl oxidase and Cu/Zn-superoxide dismutase. **Conclusions:** Overall, these results provide definitive evidence for distinct roles of MNK and WND in the human placenta, and are

consistent with a role for MNK in the transport of copper into the fetal circulation, and through delivery of copper to placental cuproenzymes, whilst WND contributes to the maintenance of placental copper homeostasis by transporting copper to the maternal circulation.

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Introduction

Copper is an essential trace element, necessary for the survival of all living organisms. It is central to the function of many copper-dependent enzymes, including lysyl oxidase and superoxide dismutase, which play a role in collagen and elastin crosslinking and as an antioxidant, respectively [1]. The importance of copper for humans is illustrated in two inherited disorders of copper metabolism, Menkes and Wilson disease. Menkes disease is a X-linked recessive disorder, caused by mutations in the gene *MNK*; *ATP7A* [2-4] leading to reduced absorption of copper from intestinal cells and defective distribution around the body, [5] resulting in deficiencies in the activity of important copper-dependent enzymes. Key features of Menkes disease include abnormal hair structure,

progressive cerebral degeneration, vascular aneurysms and thrombosis [5]. Wilson disease is an autosomal recessive disorder caused by a mutation in the Wilson gene, *WND;ATP7B* [6-8] resulting in toxic copper accumulation in the liver, and brain [5]. Patients with Wilson disease show a range of clinical conditions including chronic hepatitis, cirrhosis and neurological disturbances including movement disorders, due to the toxic accumulation of copper in the liver and brain [9].

The MNK and WND proteins are P-type ATPases responsible for the translocation of copper across cell membranes. MNK is expressed in most tissues except the liver and WND is predominantly expressed in the liver [4,6]. Despite the differences in the clinical presentation of Menkes and Wilson disease, these ATPases have been shown to have a similar intracellular functions, suggesting that the different clinical features are due to tissue specific expression. Interestingly, previous studies have shown that WND can functionally substitute for MNK in fibroblast cells [10, 11].

Copper is important during pregnancy as deficiencies or excesses of these elements can affect placental development, fetal growth and the uptake of other essential nutrients. Copper deficiency results in early embryonic death and gross structural abnormalities including skeletal, pulmonary and cardiovascular defects [12, 13]. The placenta is one of the few organs in the body that expresses both MNK and WND [4, 6]. The study of placental copper transport in fetuses with Menkes disease at mid-gestation showed high concentrations of copper in the placenta [14] and a reported case study of the copper disposition of the fetus and placenta in a female patient with untreated Wilson disease also showed that copper accumulated on the maternal side of the placenta [15]. From this it is apparent that there is a defect in placental copper transport when either the Menkes or the Wilson proteins are not functional. It is not clear whether MNK and WND participate in the directional transport of copper within human placental cells.

In addition to copper transport across the placenta to the fetus, MNK and WND may play a role in delivering copper to copper-dependent enzymes within the placenta. Both lysyl oxidase and superoxide dismutase are present in the human placenta throughout gestation [16, 17]. The enzyme lysyl oxidase catalyses the final step of collagen and elastin cross-linking [18] and superoxide dismutase plays an important role in the placenta as it catalyses the dismutation of superoxide radicals into oxygen and hydrogen peroxide, thereby protecting living cells against the harmful effects of superoxide anions [19]. Despite

what is known of the role of lysyl oxidase and superoxide dismutase in the placenta, the role of the ATPases in interacting with and delivering copper to lysyl oxidase and superoxide dismutase in the placenta remains unknown.

Our report is the first to describe a copper transport function for MNK and WND in cultured Jeg-3 placental cells. We show that MNK and WND have distinct functions in the transport of copper in opposite directions within placental cells and that MNK is the predominant transporter of copper from the basolateral membrane, whilst WND transports copper from the apical membrane. In addition, this study showed that MNK is involved in the delivery of copper to important placental cuproenzymes.

Materials and Methods

Cell Culture

Human choriocarcinoma, Jeg-3 cells were grown and maintained in EMEM (Trace, Melbourne, Australia) containing 10% (v/v) Fetal Bovine Serum (FBS) (CSL, Melbourne, Australia). The cultures were maintained at 37°C in 5% CO₂ in air. Cells were passaged when confluent using a 0.025% (v/v) Trypsin/EDTA solution (Sigma-Aldrich, Sydney, Australia).

siRNA Oligonucleotide Design and Construction

Oligonucleotides (21-mer) were designed to the *hMNK* and the *hWND* open reading frame (ORF) sequence as specified in the manufacturers protocol (Ambion siRNA Construction Kit, GeneWorks, Thebarton, Australia). Two sets of oligonucleotide sequences were selected and designated siMNK-a (AATCCTGA CCCTCTCCCTGTTCTGTCTC), siMNK-b (AAAACAGGGAGAGGGTCTAG GACCTGTCTC) and siWND-a (AATGT TGGCTATGAAGGTGGTCTGTCTC), siWND-b (AAACC ACCTTCATAGCCAACACCTGTCTC). Additionally, control siRNA oligonucleotides lacking significant homology to any human transcript were designed and designated siCont-a (AAT GCA TGT GTC ATC GTG ATA CCT GTC TC) and siCont-b (AAT ATG ACG ATG ACA CAT GCA CCT GTC TC). The 'Silencer' siRNA construction kit from Ambion (GeneWorks, Thebarton, Australia) was used to create the *hMNK*, *hWND*, and control siRNAs according to the manufacturers protocol as follows: All oligonucleotides were used for in vitro transcription with T7 RNA polymerase, then sense and antisense strands were hybridised to create double stranded RNA, the leader sequences of which were removed by digesting with single-strand specific ribonuclease and the resulting small interfering RNA (siRNA) was purified.

siRNA Transfections

Jeg-3 cells were grown in EMEM and 10% FBS in 25cm³ culture flasks to 70% confluency. A concentration of 50nM - 100nM of siRNA and 6μL/mL of oligofectamine (Invitrogen

Life Technologies, Melbourne, Australia) was prepared in OPTI-MEM 1 reduced serum medium (Invitrogen Life Technologies, Melbourne, Australia) and incubated at RT for 30 minutes. The siRNA/oligofectamine solution was added to the cells in the culture flask for 24 hours and then recovered in EMEM and 10% FBS for a further 48 hours. The cells were then collected, protein extracted and processed for western blot analysis, as described.

MNK and WND Expression Plasmids

For the over-expression of hMNK, the CMV promoter of *pCMB336* was replaced with the CAG promoter [20] and the vector was cut with SacI/XhoI restriction enzymes (Roche Diagnostics, Castle Hill, NSW). This resulted in the *pCMB344* vector [21]. For the over-expression of hWND, the CMV promoter for *pCMB336* was replaced with a CAG promoter and cut using the *BamHI* restriction endonucleases (Roche Diagnostics, Castle Hill, NSW) and resulted in the *pJFM543* vector (M. Fields, Manuscript in preparation). *E. coli* JM109 strain was transformed with the plasmids according to the manufacturers instructions. Bacterial clones containing the correct sequences were isolated, grown in LB medium and the plasmids were isolated using the QIAGEN midi-prep plasmid extraction kit (QIAGEN, Melbourne, Australia) according to the user protocol.

Stable Transfections

The hMNK and hWND plasmids were transfected into Jeg-3 cells using FuGene 6 reagent (Life Technologies, Melbourne, Australia) following the manufacturers instructions. Briefly, the Jeg-3 cells were grown in EMEM and 10% FBS in 25cm³ culture flasks to 80% confluency. A concentration of 0.1µg/µL of the DNA and 6µL/mL of FuGene 6 reagent (Invitrogen Life Technologies, Melbourne, Australia) was prepared in OPTI-MEM 1 reduced serum medium (Invitrogen Life Technologies, Melbourne, Australia) and incubated at RT for 30 minutes. The DNA/ FuGene 6 solution was added to the EMEM media on the cells in the culture flask. The cells stably expressing the hMNK and the hWND from the CAG promoter were isolated using G418 selection (Invitrogen Life Technologies, Melbourne, Australia). The Jeg-3 cells were also transfected with an empty vector as described above. The stable cells were then collected and processed for Real Time PCR and western blot analysis as described.

Total protein content

Transfected Jeg-3 cells were treated with 0.025% trypsin/EDTA solution, cell suspensions were centrifuged and cell pellets collected. Cell pellets were resuspended and homogenised in 1% (w/v) SDS in 10mM Tris-HCl pH 7.5 then sonicated for complete cell disruption. The homogenate was centrifuged at 2000g at 4°C for 10 minutes and supernatant collected. The total protein content was measured using the DC Protein Assay Kit (Bio-Rad Laboratories, California, USA) calibrated against bovine serum albumin (BSA) standards.

Western Blot Analyses

Between 30-60µg of total protein was separated by SDS/PAGE and transferred to nitrocellulose membranes (Pall Gelman, Ann Arbor, Michigan, USA). Membranes were blocked in 1% (w/v) casein in Tris Buffered Saline (TBS) for 1h at room temperature (RT). Diluted primary antibodies were applied and incubated overnight at 4°C. Polyclonal MNK and WND antibodies MNK [21] and WND, [22]) were diluted 1/1000 in 1% (w/v) casein in TBS. Membranes were rinsed and exposed to 1/2000 dilution of HRP-conjugated secondary antibody (Silenus, Melbourne, Australia) in 1% (w/v) casein in TBS for 2h at RT. After removal of excess secondary antibody, membranes were rinsed twice in TBS with 0.1% Tween 20. Proteins were detected by enhanced chemiluminescence (POD Chemiluminescence Blotting Substrate, Roche Diagnostics, NSW, Australia). To monitor protein loading, membranes were stripped in Re-Blot solution (Chemicon International, California, USA) and re-probed with monoclonal β-actin primary antibody (Sigma-Aldrich, Sydney, Australia) diluted 1/5000. Lysates prepared from the breast cancer cell line PMC42 were used as a positive control for MNK and WND proteins [23]. Densitometry to quantify results was performed using Fuji Film Multi Gauge V2.3 computer software.

Real Time PCR

Total cellular RNA was extracted from Jeg-3 cells using an RNeasy Mini Kit (Qiagen, Victoria, Australia). cDNA was synthesized with random primers (Roche, NSW, Australia) and reverse transcriptase. Amplification reactions were performed with 1 x SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK), 3µM of forward and reverse primers for *hMNK* (TCG GAG GCT GGT ACT TCT ACA TTC, CAA AGA GTA GGC AAA TGC AAT GG) and *hWND* (CAC GAA AGC CAA TTT CCT CAA T, CCAGCAAAG CCC TTG TTAAGT T) and 20ng cDNA. Samples were analysed in triplicate in 20µL total volume using GeneAmp 5700 Sequence Detection System (PE Biosystems, Forster City, CA). An internal control of GAPDH was used to as a control for RNA quantities and the efficiency of reverse transcription. The abundance of each mRNA measured as the threshold cycle value (CT), was calculated after each reaction. The relative RNA expression level of each sample was calculated using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ was the difference between the control ΔCT and the treatment ΔCT .

⁶⁴Cu Transport Assay

Stable over-expressing Jeg-3 cells and transiently transfected siRNA Jeg-3 cells were grown to confluency on Engelbreth-Holm-Swarm (EHS)-matrix coated (diluted 1:10 in mH₂O, Sigma-Aldrich, Sydney, Australia) PET track-etched/porous membrane cell culture inserts with 1mL of EMEM in the top chamber and 2mL of EMEM in the bottom chamber. Radioactive copper (⁶⁴Cu) was purchased from ARI, National Medical Cyclotron, Australian Nuclear Science and Technology Organisation, NSW, Australia. The average radioactive

Fig. 1. MNK and WND protein and mRNA levels in siRNA transfected Jeg-3 cells. Western blot analysis of MNK (A) or WND (C) protein levels in Jeg-3 cells transfected with 50nM siRNA (Lane 2), 100nM siRNA (Lane 3) or control siRNA (Lane 1). β -actin is shown below indicating equal loading. Bar graph of the relative MNK (B) or WND (D) mRNA expression level determined by real time PCR with GAPDH as the internal control. Asterisks denotes significant difference ($p < 0.001$).

concentration of ^{64}Cu was 2.6GBq/ml of copper in the form of CuCl_2 diluted in 0.1M HCl to final concentration of 0.15 μM . One to 2 microlitres of ^{64}Cu /ml was added to the media and this media was added to either the top (apical) chamber or the bottom (basolateral) chamber. Media aliquots were taken from the opposite chamber to where the ^{64}Cu had been added (either the apical or the basolateral side), after 0.5 hour, 1 hour, 2 hours, 4 hours and 6 hours incubation. These aliquots were counted using a Minaxi Auto Gamma counter.

Accumulation of ^{64}Cu Assay

Over-expressing and siRNA transfected Jeg-3 cells were grown to confluency on EHS-matrix coated (diluted 1:10 in mH_2O , Sigma-Aldrich, Sydney, Australia) PET track-etched/porous membrane cell culture inserts. The growth media was supplemented with 1.0 μL – 2.0 μL ^{64}Cu /mL and incubated for 6 hours as described in the previous section. After 6 hours the membranes were rinsed with cold HBSS and solubilised in 0.2M KOH. The cell-associated radioactivity was measured with a Minaxi Auto Gamma gamma counter. Copper accumulation was normalized to the protein concentration of the cell lysate (quantified using a Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, California, USA).

Lysyl Oxidase Assay

The lysyl oxidase assay was adapted from an existing assay [24]. Jeg-3 cells were grown to 70% confluency in EMEM and 10% FBS in 25cm³ flasks for 24 hours and then transfected with siRNA (MNK, WND and hCTR1) as described. Cells were allowed to recover in EMEM and 10% FBS for 24 hours and then TMEM (serum free, phenol red-free media) was added to the cells. After a further 24hrs the media was collected and centrifuged for 10 mins at 10000 g at 4°C. The hMNK or hWND stably-transfected cells were also grown to 70% confluency and then TMEM (serum free, phenol red-free media) was added to the cells, then after a further 24hrs the media was collected and centrifuged for 10 mins at 10,000x g at 4°C. Samples were then prepared in a final volume of 2mL containing 1.2M urea, 0.05M sodium borate (pH 8.2) (Sigma-Aldrich, Sydney, Australia), 1 unit/ml horseradish peroxidase (Sigma-Aldrich, Sydney, Australia), 10 μM Amplex Red (Molecular Probes, Oregon, USA) and 10mM 1,5-diaminopentane (Sigma-Aldrich, Sydney, Australia). A parallel set of solutions were prepared with 500 μM β -aminopropionitrile (BAPN, Sigma-Aldrich, Sydney, Australia), which inhibits lysyl oxidase activity. The samples were incubated at 37°C for 30 mins and the fluorescence was measured using a Labsystems Multiscan Plate Reader and

Fig. 2. MNK and WND protein and mRNA levels in over-expressing Jeg-3 cells. Western blot analysis of MNK (A) or WND (C) protein levels in Jeg-3 cells over-expressing MNK or WND (Lane 2) and control Jeg-3 cells (Lane 1). β -actin is shown below indicating equal loading. Bar graph of the relative MNK (B) or WND (D) mRNA expression level determined by real time PCR with GAPDH as the internal control. Asterisks denotes significant difference ($p < 0.001$).

Genesis Lite 3.03 with excitation and emission wavelengths at 563nm and 587nm. The amount of hydrogen peroxide produced by the action of lysyl oxidase was determined by comparing fluorescence changes to that of a standard curve prepared using hydrogen peroxide solutions (0nmol-1.5nmol) (Sigma-Aldrich, Sydney, Australia). The units of enzyme activity were given as nmol of hydrogen peroxide produced/ above BAPN control.

Superoxide Dismutase Assay

The superoxide dismutase enzymatic assay was performed using a superoxide dismutase assay kit (Dojindo Molecular Technologies Inc., Maryland, USA). This method utilises a highly water-soluble tetrazolium salt that produces a water-soluble formazan dye upon reduction with a superoxide anion and further producing a colourimetric reaction. Jeg-3 cells were grown to 70% confluency in EMEM and 10% FBS in 25cm³ flasks for 24 hours and then transfected with siRNA (MNK, WND or hCTR1) as described. Stable Jeg-3 cells over-expressing hMNK or hWND were also grown to confluence. Both the siRNA transfected and the hMNK/hWND cells were collected, total protein was extracted and quantified as described (Dojindo Molecular Technologies Inc., Maryland, USA). The superoxide dismutase assay was performed as specified in the manufacturer's protocol. Fifty micrograms/ μ L of protein extract in 1% (w/v) SDS in 10mM Tris-HCl was used

in each enzymatic reaction to ensure an equal amount of protein in each reaction and the absorbance of the colourimetric result of this reaction was read at 450nm using a Labsystems Multiscan Plate Reader and Genesis Lite 3.03. The percentage superoxide dismutase activity within the mixture was calculated. The reactions were repeated, adding 5 μ M KCN to the samples to inhibit Cu/Zn superoxide dismutase activity, leaving the percentage of Mn superoxide dismutase activity expressed as a percentage of the total superoxide dismutase activity. This Mn superoxide dismutase activity (%) was subtracted from the total superoxide dismutase activity (%), resulting in the total Cu/Zn superoxide dismutase (%) activity. This percentage activity was graphed using Microsoft Excel Software.

Statistics

Data are presented as means $\pm$ SD. Statistical analyses were carried out using a paired t-test with unequal variance.

Results

siRNA transfection decreased the levels of MNK and WND in Jeg-3 cells

To determine whether MNK or WND expression was reduced in Jeg-3 cells transfected with siRNA,

Fig. 3. Transport and Accumulation of Cu (Apical to Basolateral) in Jeg-3 cells. To measure apical to basolateral Cu transport, Jeg-3 cells were grown as a polarised monolayer and ^{64}Cu was added at the apical chamber of the cells. Line graphs of the pmole Cu/ μg protein from the apical chamber to the basolateral chamber, per hour, in Jeg-3 cells transfected with control, MNK or WND siRNA (A and A') or in Jeg-3 cells over-expressing MNK or WND (C). Bar graphs of the intracellular Cu accumulation in Jeg-3 cells transfected with control MNK or WND siRNA (B) or over-expressing MNK or WND (D). Asterisks denotes significant difference ($p < 0.001$).

western blot analysis was carried out (Figure 1). The protein levels of MNK relative to β -actin, as determined by densitometry, were decreased in extracts from cells transfected with 100nM of MNK siRNA (Figure 1A, lane 2), to 0.35 ± 0.09 of the levels of extracts from cells transfected with 100nM of control siRNA (Figure 1A, lane 1). Similarly, there was a significant decrease of 0.55 ± 0.11 in the relative *MNK* mRNA levels in Jeg-3 cells transfected with 100nM of siRNA, as determined by real time PCR analysis, when compared with the control Jeg-3 cells (Figure 1B) ($p < 0.01$; t-test). The level of the WND protein relative to β -actin, as determined by densitometry, was decreased to 0.37 ± 0.14 in the extracts from cells transfected with either 50nm or 100nM of WND siRNA (Figure 1C, lanes 2 and 3 respectively), compared with extracts from cells transfected with control

siRNA (Figure 1C, lane 1) ($p < 0.01$; t-test). The *WND* mRNA levels were also decreased to 0.73 ± 0.07 in cells transfected with WND siRNA compared with the control cells (Figure 1D) but this decrease was not significant. These results are from three independent siRNA transfections, each conducted in triplicate.

Over-expression of MNK and WND in Jeg-3 cells

Jeg-3 cells were transfected with either MNK expression vector *pCMB344* or the WND expression vector *pJFM543*. Stable clones were selected using G418. The levels of over expression were detected by Western blot analysis and Real Time PCR (Figure 2). MNK protein relative to β -actin, as determined by densitometry, were increased by 2.6-fold (± 1.2) when compared with control cells (Figure 2A, lanes 2 and 1).

Fig. 4. Transport and Accumulation of ^{64}Cu (Basolateral to Apical) in Jeg-3 cells. To measure basolateral to apical Cu transport, Jeg-3 cells were grown as a polarised monolayer and ^{64}Cu was added at the basolateral chamber of the cells. Line graphs of the pmole Cu/μg protein from the basolateral chamber to the apical chamber, per hour, in Jeg-3 cells transfected with control, MNK or WND siRNA (A) or over-expressing MNK or WND (C). Bar graphs of the intracellular Cu accumulation in Jeg-3 cells transfected with control, MNK or WND siRNA (B) or over-expressing MNK or WND (D). Asterisks denotes significant difference ($p < 0.001$).

The *MNK* mRNA levels were significantly increased by 6.2-fold (± 0.31) in the over-expressing Jeg-3 cells, compared with cells transfected with the control plasmid (Figure 2B) ($p < 0.01$; t-test). The expression of WND

Fig. 5. Effect of MNK and WND expression levels on lysyl oxidase or superoxide dismutase activity. Bar graphs of lysyl oxidase activity in Jeg-3 cells transfected with control, MNK or WND siRNA (A) or over-expressing MNK or WND (B). Asterisks denotes significant difference ($p < 0.001$). Bar graphs of percentage Cu/Zn superoxide dismutase activity in Jeg-3 cells transfected with control, MNK or WND siRNA (C) or over-expressing MNK or WND (D). Asterisks denotes significant difference ($p < 0.001$).

protein levels in Jeg-3 cells transfected with WND expression plasmid were increased by 3.4-fold (± 0.47) when compared with the control cells (Figure 2C, lanes 2 and 1), and *WND* mRNA levels were also significantly

increased by 26-fold (± 0.57) (Figure 2D) ($p < 0.001$; t-test).

Reduction of MNK resulted in a decrease in the efflux of Cu from the basolateral membrane of the Jeg-3 cells

The transport of copper from the apical chamber to the basolateral chamber and vice versa was investigated using ^{64}Cu . After 1 hour, 2 hours, 4 hours and 6 hours, the efflux of copper from the basolateral membrane was significantly decreased by 8-fold (± 0.09), 9-fold (± 0.07), 2.8-fold (± 0.02) and 6-fold (± 0.04) respectively in Jeg-3 cells transfected with MNK siRNA, relative to the Jeg-3 cells transfected with control siRNA (Figure 3A) ($p < 0.001$; t-test). There was also a 8-fold (± 0.17) decrease in the efflux of copper from the basolateral membrane after 1 hour in the Jeg-3 cells transfected with WND siRNA (Figure 3A). The data shown that rate of copper efflux was reduced in the siRNA cells relative to the control at 30 minutes and one hour (Figure 3A') but was similar in the time period between 2 and 6 hours, as indicated by the slope of the graph. The MNK siRNA transfected cells also accumulated significantly more intracellular copper, when copper was added to the apical chamber (Figure 3B) ($p < 0.01$; t-test). WND siRNA had no effect on copper accumulation.

Increased expression of MNK resulted in an increase in the efflux of copper from the basolateral membrane of the Jeg-3 cells

Over-expression of MNK resulted in a significant increase in copper transport from the apical to the basolateral chamber at all time points (Figure 3C) ($p < 0.01$; t-test). In contrast over-expression of WND did not affect copper transport in this direction (Figure 3C). The accumulation of copper in cells over-expressing either MNK or WND was half that of the control Jeg-3 cells (Figure 3D) ($p < 0.01$; t-test).

Decreased expression of WND resulted in a decrease in the efflux of copper at the apical membrane of the Jeg-3 cells

To measure copper efflux at the apical membrane of the Jeg-3 cells, ^{64}Cu was added to the basolateral chamber. In cells transfected with WND siRNA efflux of copper from this membrane was significantly decreased by 5-fold (± 0.04) (Figure 4A) ($p < 0.001$; t-test), and there was also a 2-fold (± 0.01) decrease in the efflux of copper from the apical membrane in the cells transfected with MNK siRNA (Figure 4A) ($p < 0.01$; t-

test). The accumulation of intracellular copper was not significantly increased in cells transfected with WND siRNA, however, MNK siRNA had no significant affect on copper accumulation (Figure 4B).

Increased expression of MNK resulted in a decrease in the efflux of copper at the apical membrane of the Jeg-3 cells

The efflux of copper from the apical membrane was significantly decreased in Jeg-3 cells over-expressing MNK ($p < 0.001$; t-test), however, there was no change in the transport of copper in cells over-expressing WND (Figure 4C). When ^{64}Cu was added to the basolateral chamber, the accumulation of intracellular copper was decreased in cells over-expressing either MNK or WND (Figure 4D), however, this decrease was not significant. These ^{64}Cu experiments were conducted in duplicate, over 3 independent investigations.

MNK expression is required for the activity of lysyl oxidase in Jeg-3 cells

To investigate the relationship between MNK and WND expression levels and the activity of the cuproenzyme, lysyl oxidase, the enzyme assay was performed in extracts from Jeg-3 cells transfected with MNK or WND siRNA or Jeg-3 cells over-expressing either MNK or WND. The results are shown in Figure 5A and demonstrate that lysyl oxidase activity was significantly ($p < 0.001$; t-test) decreased in cells where MNK levels were decreased by siRNA treatment, when compared with the control cells. There was no significant effect on the activity of lysyl oxidase in extracts from Jeg-3 cells transfected with WND siRNA (Figure 5A). In contrast to the siRNA studies, the activity of lysyl oxidase was significantly increased by 3-fold (± 0.02) in Jeg-3 cells over-expressing MNK ($p < 0.01$; t-test), however, there was no corresponding increase in the activity of lysyl oxidase in the Jeg-3 cells over-expressing WND (Figure 5B).

Decreased expression of MNK resulted in a decrease in the activity of Cu/Zn superoxide dismutase in Jeg-3 cells

Cu/Zn superoxide dismutase activity was significantly decreased by 15% in cells transfected with MNK siRNA as compared with the control siRNA cells (Figure 5C) ($p < 0.01$; t-test). Cu/Zn superoxide dismutase activity was also reduced in cells transfected with siRNA for WND, however this decrease was not significant

(Figure 5C). The levels of Cu/Zn superoxide dismutase activity were not significantly altered in Jeg-3 over-expressing either MNK or WND (Figure 5D).

Discussion

The adverse effects of copper deficiency on fetal development illustrate the importance of copper during pregnancy. Given that copper accumulates in the placenta in both Menkes and Wilson disease [14, 15] and that we previously detected both MNK and WND in human placenta [23], we predicted a role for both proteins in the placental transport of copper. As it is known from previous studies showing that in fibroblasts WND can substitute the function of MNK, the question of whether these proteins have distinct roles *in vivo* is not clear.

This study is the first to report the vectorial transport of copper by the MNK and WND proteins in human Jeg-3 placental cells. A role for MNK in placental copper transport was previously shown using placental BeWo cells, where the efflux of ^{67}Cu from these cells was absent in cells not expressing MNK, and a functional efflux system was restored with the expression of the protein [25], suggesting a link between the expression of the MNK gene product and the capacity of the BeWo cells to release copper ions.

The ^{64}Cu transport data reported in this investigation demonstrated that when MNK protein levels were increased through over-expression, there was a corresponding increase in the efflux of copper from the basolateral membrane. This indicates that MNK functions in the efflux of copper from the basolateral membrane of placental cells. Further substantiation of this is seen through the correlation between reduced MNK expression through siRNA transfection and a corresponding decrease in the efflux of copper from this membrane. The data show that the rate of decrease of copper efflux in knockdown cells occurred only up to one hour and that after this time point the rates of copper efflux were similar in MNK and WND knockdown cells and in control cells. A possible explanation for this is that in cells where MNK and WND were knocked down, there was reduced capacity for cells to efflux copper, as MNK and WND are the major copper efflux molecules. Consequently to balance this reduced efflux, the cell reduced the uptake of copper, possibly by internalization of CTR1. The ^{64}Cu medium added to the cells contained $0.15\mu\text{M}$ copper, which is higher concentration than that of the culture medium in which the cells were grown. When the ^{64}Cu

medium was added to the cells, initially over the first hour, the MNK and WND knockdown cells had a reduced capacity to transport copper compared to the control, as CTR1 would be internalised and MNK and WND activity was reduced by knockdown. After one hour the MNK and WND knockdown cells adjusted the balance of uptake and efflux to maintain copper homeostasis.

Previous localisation studies have shown that MNK was present at the basal surface of the syncytiotrophoblast and the cytotrophoblast cells [23]. The ^{64}Cu data presented here is also consistent with the function of MNK in mediating copper absorption from the gastrointestinal tract where the protein was located at basolateral membrane of enterocytes [26] and at the basolateral membrane of MDCK (kidney) cells [27].

Cellular copper accumulation, measured after 6 hours of exposure to ^{64}Cu , provided direct evidence of a link between copper efflux and the protein levels of MNK, as the intracellular accumulation of copper was reduced by 2-fold in Jeg-3 cells in which MNK protein levels had been increased by 2.6-fold. This was seen only when ^{64}Cu was added to the apical, not the basolateral, chamber and confirms the hypothesis that MNK functions primarily to efflux copper from the basolateral membrane. Further support is provided by results showing an increase in the accumulation of copper in Jeg-3 cells where MNK expression was decreased. The cell culture results are consistent with observations *in vivo* showing firstly an increased accumulation of copper in placenta of babies born with Menkes disease [28]. Also, in a mouse model of Menkes disease, the brindled mutant mouse, MNK was involved in the transport of copper to the fetus, as placental copper content was increased and a direct block of placental transport was demonstrated in this mouse model [29, 30]. Taken together, these data provide strong evidence that MNK functions to efflux copper from the basolateral membrane to the fetal circulation.

Observations show that when copper was added to the basolateral chamber, the copper retention was decreased in cells overexpressing WND, but the export of copper to the apical chamber was not affected. An explanation for this might be that while in fully polarized cells *in vivo*, WND effluxes copper from the apical surface of cells and MNK effluxes copper from the basolateral surface, JEG cells in culture, however, are not fully polarized and therefore MNK and WND are not completely localized to the apical and basolateral surfaces and may be active at both domains.

An important function for MNK in the delivery of copper to copper-dependent enzymes in placental cells is

shown by the results of the lysyl oxidase assay. Lysyl oxidase is vital for the structure of the placenta by catalyzing the final step of collagen and elastin cross-linking [18], to provide tensile strength and elasticity. We found a correlation between MNK expression levels and lysyl oxidase activity, where reduced levels of MNK decreased lysyl oxidase activity. Previous studies have shown lysyl oxidase activity is sensitive to changes in copper status and decreased functional activity of the enzyme is found in disorders such as Menkes and Wilson disease [31, 32]. Our data provides conclusive evidence for the role of MNK in the delivery of copper to lysyl oxidase and hence placental function.

A decrease in MNK expression resulted in a reduction in the activity of Cu/Zn superoxide dismutase, an enzyme that is important in protecting the fetus from superoxide anion radicals [33]. This decreased in Cu/Zn superoxide dismutase activity occurs despite the fact that there is an increase in intracellular copper accumulation when MNK expression is reduced. This is an interesting result which suggests that the activity of this enzyme is unaffected by the rise in intracellular copper levels and is instead influenced by the expression levels of MNK. It is possible that copper accumulation induces metallothionein which reduces the availability of copper to the enzyme. From our results it is clear that the levels of MNK in Jeg-3 placental cells is directly correlated with the activity of Cu/Zn superoxide dismutase and this may have clinically relevant consequences for fetal and placental development. The relationship between MNK and the activity of and Cu/Zn superoxide dismutase warrants further investigation.

Based on the different intracellular localisations of MNK and WND found previously [23], it was hypothesized that both MNK and WND have different roles in copper transport in the placenta. This hypothesis has been confirmed by the ^{64}Cu studies presented here, where a reduction in WND expression was associated with a corresponding decrease in the efflux of copper from the apical membrane, but only when ^{64}Cu was added to the basolateral chamber. This is in contrast to the copper transport function of MNK, where changes in MNK expression altered the efflux of ^{64}Cu only at the basolateral membrane of the Jeg-3 cells. This provides the first evidence demonstrating that MNK and WND efflux copper from opposite membranes in polarized Jeg-3 cells.

Additional evidence for the function for WND in copper efflux from the apical membrane is suggested by the presence of WND at the sub-apical membrane in

hepatocytes cells in the liver. In these cells, WND was localised at the *trans*-Golgi network in basal copper conditions and when cellular copper levels increased, the protein moved from the *trans*-Golgi network, but unlike MNK it relocated to a vesicular compartment towards the hepatocyte canalicular (apical) membrane [34, 35]. The accumulation of copper in the liver of patients with Wilson disease suggests that this protein serves an important function in the efflux of copper from hepatocytes into bile and thus the maintenance of copper homeostasis. In the placenta WND may have a similar role, by transporting copper towards the maternal circulation, to prevent the accumulation of copper within placental cells, as there is a copper concentration gradient that favours the transport of copper towards the fetal circulation [36].

It was interesting to note that when the expression of WND was increased, there was no corresponding increase in the efflux of copper from the apical membrane. This suggests that there may be a mechanism in placental cells, whereby an increase in this protein does not result in an overall increase in the efflux of copper at the apical membrane, to ensure that there is sufficient intracellular copper levels for transport to the developing fetus. In addition, it was shown that an increase in WND resulted in an overall decrease in the accumulation of copper, when ^{64}Cu was added to the basolateral chamber, despite the fact that there was no corresponding increase in copper efflux at the apical membrane. This may suggest that MNK is functioning to efflux copper from the basolateral membrane, as a mechanism to prevent an increase in intracellular copper accumulation. Further investigations are required to determine the intracellular mechanisms regulating the function of these copper transporters.

Further information of the role of WND within the placenta is suggested by our data showing that changes in the expression of WND within the Jeg-3 cells did not alter the activity of either lysyl oxidase or Cu/Zn superoxide dismutase. This suggests that WND does not interact with these cuproenzymes, but rather it may primarily function to transport copper towards the maternal circulation and thus maintain intracellular copper homeostasis. It is also possible that WND has a function in copper delivery to ceruloplasmin, which is expressed in the placenta [37], and therefore plays a role in delivery of iron to the fetus. Such a function has been proposed in studies using BeWo choriocarcinoma cells, which express a ferroxidase with similar immunoreactivity to ceruloplasmin [38].

In summary, our data show that MNK and WND

have specific and non-overlapping copper transport roles in the human placenta. MNK and WND were found to efflux copper from opposite membranes of the placental Jeg-3 cells. While MNK transported copper across the basolateral membrane, consistent with a role for this protein in the delivery of copper towards the fetal circulation, WND functioned to efflux copper from the apical membrane, suggesting that it does not play a role in the transport of copper to the developing fetus, but rather to the maternal circulation, to prevent excess copper transport to the fetus. Decreased expression of MNK significantly decreased the activity of lysyl oxidase and Cu/Zn superoxide dismutase, suggesting a role for MNK

in the delivery of copper to these enzymes. Interestingly, WND expression had no effect on the levels of these enzymes within the Jeg-3 cells. In conclusion, these data establish that MNK and WND have distinct functional roles in the efflux of copper from human placental cells.

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