Hormonal regulation of the Menkes and Wilson copper-transporting ATPases in human placental Jeg-3 cells

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Copper deficiency during pregnancy results in early embryonic death and foetal structural abnormalities including skeletal, pulmonary and cardiovascular defects. During pregnancy, copper is transported from the maternal circulation to the foetus by mechanisms which have not been clearly elucidated. Two copper-transporting ATPases, Menkes (ATP7A; MNK) and Wilson (ATP7B; WND), are expressed in the placenta and both are involved in placental copper transport, as copper accumulates in the placenta in both Menkes and Wilson disease. The regulatory mechanisms of MNK and WND and their exact role in the placenta are unknown. Using a differentiated polarized Jeg-3 cell culture model of placental trophoblasts, MNK and WND were shown to be expressed within these cells. Distinct roles for MNK and WND are suggested on the basis of their opposing responses to insulin. Insulin and oestrogen increased both *MNK* mRNA and protein levels, altered the localization of MNK towards the basolateral

membrane in a copper-independent manner, and increased the transport of copper across this membrane. In contrast, levels of WND were decreased in response to insulin, and the protein was located in a tight perinuclear region, with a corresponding decrease in copper efflux across the apical membrane. These results are consistent with a model of copper transport in the placenta in which MNK delivers copper to the foetus and WND returns excess copper to the maternal circulation. Insulin and oestrogen stimulate copper transport to the foetus by increasing the expression of MNK and reducing the expression of WND. These data show for the first time that MNK and WND are differentially regulated by the hormones insulin and oestrogen in human placental cells.

Key words: Copper, insulin, Menkes disease, oestrogen, pregnancy, Wilson disease.

INTRODUCTION

Copper is an essential trace element, necessary for the survival of all living organisms. It is central to the function of many enzymes, including cytochrome *c* oxidase, lysyl oxidase and superoxide dismutase, forming an integral part of the enzymes' active site. The importance of copper for humans is illustrated by 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* [1–3]. The reduced absorption of copper from intestinal cells and defective distribution around the body [4] in this disease leads to reduction in the activity of important copper-dependent enzymes [5]. Key features of Menkes disease include abnormal hair structure, progressive cerebral degeneration, vascular aneurysms and thrombosis [4]. 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 [4]. Patients with Wilson disease show a range of clinical conditions including chronic hepatitis, cirrhosis and neurological disturbances including movement [4].

That MNK is important for normal development is evidenced in babies with Menkes disease. Due to copper deficiency, Menkes babies are lethargic, have sparse hair and develop hypothermia and convulsions, leading to death within the first year of life [9,10]. Evidence suggests that MNK is involved in the transport of copper to the foetus, as an increase in the placental copper content is observed in a mouse model of Menkes disease, the

brindled mouse, and a direct block of placental transport has been demonstrated in this mouse model [11,12]. The role of WND in early development is not clear, but as copper accumulates in the placenta during gestation in both Menkes and Wilson disease [13,14] a role for both copper ATPases in the transfer of copper across the placenta to the foetus is likely.

In a previous study we were the first to demonstrate that both MNK and WND are expressed in the human placenta and are localized differentially within the syncytiotrophoblast layer [15]. However, nothing is known about the regulation of these proteins in placental tissue. Hormonal pathways are involved in the regulation of some placental transporters, for example insulin treatment resulted in a dose-dependent increase in the transport of glucose in human first-trimester placental trophoblast cells, which correlated with an insulin-induced increase in GLUT (glucose transporter) 1 mRNA [16]. IGFs (insulin-like growth factors)-I and -II also enhanced glucose uptake in human first-trimester trophoblasts [17]. In addition, studies using primate placenta have also found that the hormone oestrogen stimulates the receptor-mediated uptake of low-density lipoprotein by increasing the levels of the low-density lipoprotein receptor mRNA levels in a cultured syncytiotrophoblast fraction of the placenta [18]. From this it is evident that hormones and growth factors such as insulin, IGF-I, IGF-II and oestrogen regulate the transport of nutrients within the placenta and may also regulate copper transport.

The placenta is one of the few tissues in the body to express both MNK and WND [6]. Given this, it allows us to study the role of both proteins to determine whether they have independent

Abbreviations used: BCS, bathocuproinedisulfonic acid; CT, threshold cycle; EHS, Engelbreth Holm-Swarm; ERE, oestrogen response elements; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; IGF, insulin-like growth factor; IRS, insulin response sequence; MNK/ATP7A, Menkes-disease protein; PET, polyethylene terephthalate; TBS, Tris-buffered saline; TGN, *trans*-Golgi network; WND/ATP7B, Wilson-disease protein.

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functions within this tissue and whether they are differentially regulated. The present study is the first to examine the role of the important gestational hormones insulin, IGF-I and IGF-II, oestrogen and progesterone in regulating the expression and localization of MNK and WND in placental cells, using polarized Jeg-3 cells as a model. In addition, the effect of hormones on the transport of copper in Jeg-3 cells was also investigated. The data presented in the present paper indicates a central role for hormones in regulating the expression of MNK and WND and suggests that these proteins may have distinct roles in transporting adequate supplies of copper to the foetus during gestation.

EXPERIMENTAL

Cell culture

Jeg-3 cells were grown and maintained in TMEM (serum-free medium) and EMEM (Eagle Minimum Essential Medium; Trace). 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). To induce cellular differentiation, Jeg-3 cells were grown to 90% confluency on PET (polyethylene terephthalate) track-etched/porous membrane cell culture inserts (0.4 µm pore size) [19]. Polarization was confirmed previously using markers for the apical and basolateral membranes. Cells were treated with insulin (2.5 ng/ml, 5 ng/ml, 10 ng/ml or 20 ng/ml; Sigma–Aldrich), IGF-I (180 ng/ml; Pathtech), IGF-II (1100 ng/ml; Pathtech), oestrogen (0.7 ng/ml, 1.35 ng/ml, 2.7 ng/ml or 5.4 ng/ml; Sigma–Aldrich) or progesterone (0.015 ng/ml; Sigma–Aldrich) for 24 h or 3 days. Cells were also exposed to 100 µM of the copper chelator BCS (bathocuproine-disulfonic acid; Sigma–Aldrich) for 24 h prior to and during hormone treatments. The cells grown on the PET track-etched/porous membrane cell culture inserts (0.4 µm pore size) were processed for immunofluorescence or harvested for RNA and protein analysis.

Total protein content

Confluent Jeg-3 cells were treated with 0.025% trypsin/EDTA solution, cell suspensions were centrifuged (2000 g for 5 min at 25°C) and cell pellets collected. Cell pellets were resuspended and homogenized in 1% (w/v) SDS in 10 mM Tris/HCl (pH 7.5), then sonicated for complete cell disruption. The homogenate was centrifuged at 2000 g at 4°C for 10 min and supernatant collected. The total protein content was measured using the DC Protein Assay Kit (Bio-Rad) calibrated against BSA standards.

Western blot analyses

Between 30 and 60 µg of total protein was separated by SDS/PAGE and transferred to nitrocellulose membranes (Pall Gelman). Membranes were blocked in 1% (w/v) casein in TBS (Tris-buffered saline) for 1 h at room temperature (25°C). Diluted primary antibodies were applied and incubated overnight at 4°C. Polyclonal MNK and WND antibodies, MNK (R17) [20] and WND (NC36) [21], were diluted 1/1000 in 1% (w/v) casein in TBS. Membranes were rinsed and exposed to 1/2000 dilution of HRP (horseradish peroxidase)-conjugated secondary antibody (Silenus) in 1% (w/v) casein in TBS for 2 h at room temperature. After removal of excess secondary antibody, membranes were rinsed twice in TBS containing 0.1% Tween 20. Proteins were detected by enhanced chemiluminescence (POD Chemiluminescence Blotting Substrate, Roche Diagnostics). To monitor protein loading, membranes were stripped in Re-Blot solution

(Chemicon) and re-probed with a monoclonal β-actin primary antibody (Sigma–Aldrich) diluted 1/5000. Lysates prepared from the breast cancer cell line, PMC42, were used as a positive control for MNK and WND proteins. Densitometry to quantify results was performed using Fuji Film Multi Gauge V2.3 computer software and ratios for protein levels were calculated relative to β-actin controls.

Real-time PCR

Total cellular RNA was extracted from Jeg-3 cells using an RNeasy mini kit (Qiagen). cDNA was synthesized with random primers (Roche) and reverse transcriptase. Amplification reactions were performed with 1 × SYBR® Green PCR Master Mix (Applied Biosystem), 3 µM of forward and reverse primers for *hMNK* (5'-TCGGAGGCTGGTACTTCTACATTC-3' and 5'-CAAAGAGTAGGCAAATGCAATGG-3') and *hWMD* (5'-CACGAAAGCCAATTTCTCAAT-3' and 5'-CCAGCAAAGC-CCTTGTTAAGTT-3') and 20 ng of cDNA. Samples were analysed in triplicate in a total volume of 20 µl using the GeneAmp 5700 Sequence Detection System (PE Biosystems). An internal control of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control for RNA quantities and the efficiency of reverse transcription. The abundance of each gene measured as the CT (threshold cycle) value, was calculated after each reaction. The relative RNA expression level of each sample was calculated using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the difference between the control ΔCT and the treatment ΔCT .

Indirect immunocytochemistry

Jeg-3 cells grown until confluent were rinsed three times with PBS, fixed in 4% (w/v) paraformaldehyde in PBS for 10 min and permeabilized with 5% (v/v) Triton X-100 (Sigma–Aldrich) in PBS for 5 min. Cells were blocked in 3% BSA in PBS for 1 h at room temperature and incubated overnight at 4°C with antibodies against MNK (R17; diluted 1/50), WND (NC36; diluted 1/10000), p230 TGN (*trans*-Golgi network) marker (diluted 1/250; Sigma–Aldrich). Alexa Fluor® 488 fluorescent-tagged secondary antibodies (diluted 1/1000; Molecular Probes) were applied for 2 h. After washing off excess secondary antibodies, ethidium bromide (diluted 1/10000) was added for 3 min to enable visualization of the nuclei. Cells were rinsed again with PBS and mounted in Antifade reagent (Bio-Rad). Cells were viewed using a Leica TCS SP2 AOBs laser confocal microscope (Leica), using oil immersion and a 100X objective. Images were captured using a Leica TCS SP2 laser, and viewed on a HP workstation with Leica Microsystems TCS SP2 software.

⁶⁴Cu transport assay

Jeg-3 cells were grown to confluency on EHS (Engelbreth Holm-Swarm)-matrix coated (diluted 1:10 in water; Sigma–Aldrich) PET track-etched/porous membrane cell culture inserts and treated with either insulin (10 ng/ml) or oestrogen (2.7 ng/ml) for 3 days. Radioactive copper (⁶⁴Cu) was purchased from ARI, Australian Nuclear Science and Technology Organisation, NSW, Australia. The average radioactive concentration of ⁶⁴Cu was 2.61 GBq/ml of copper in the form of CuCl₂ diluted in 0.1 M HCl. To the medium, 1–2 µl of ⁶⁴Cu/ml was added and this medium was added to either the apical or the basolateral chamber. Media aliquots were taken from the opposite chamber to where the ⁶⁴Cu had been added (either the apical or the basolateral side), after 1 h, 2 h, 4 h and 6 h. Aliquots were counted using a Minaxi Auto Gamma counter.

Accumulation of ^{64}Cu assay

Jeg-3 cells were grown to confluency on EHS-matrix coated (diluted 1:10 in water; Sigma–Aldrich) PET track-etched/porous membrane cell culture inserts and treated with either insulin (10 ng/ml) or oestrogen (2.7 ng/ml) for 3 days. The growth medium was supplemented with 1–2 μl of ^{64}Cu /ml and incubated for 6 h as described. After 6 h the membranes were rinsed with cold HBSS (Hanks balanced salt solution) and solubilized with 0.2 M potassium hydroxide. The cell-associated radioactivity was measured with a Minaxi Auto Gamma counter. Copper accumulation was normalized to the protein concentration of the cell lysate (quantified using a Bio-Rad Protein Assay Kit, Bio-Rad).

ClustalX® and alignment

The promoter sequences for *hMNK* and *hWND* were located using the ncbi-website (<http://www.ncbi.nlm.nih.gov>) and alignments with human IRSs (insulin response sequences) and human EREs (oestrogen response elements) were performed using ClustalX® alignment software.

Statistics

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

RESULTS

Western blot analysis to detect MNK and WND protein in Jeg-3 cells

To determine whether MNK and WND were present in Jeg-3 placental cells, Western blot analysis was performed on cell extracts. A band of approx. 180 kDa in size, consistent with the size of MNK [22], was detected in Jeg-3 cell extracts (Figure 1A, lane 2) and PMC42 cells (Figure 1A, lane 1) which was used as a positive control. Bands of about 165 kDa in size, consistent with the expected size of WND [23], were also detected in Jeg-3 cells and PMC42 cells (Figure 1B, lanes 2 and 1 respectively).

Immunolocalization of MNK and WND in Jeg-3 cells

Immunofluorescence was used to investigate the intracellular localization of MNK and WND in the polarized Jeg-3 cells. As seen in Figure 1(C), MNK (green) showed a cytoplasmic localization with significant co-localization with the p230 TGN marker (red) in the perinuclear region (orange; merge). Figure 1(D) shows that WND had a predominantly cytoplasmic distribution (green) and co-localized with the p230 TGN marker (red) in the perinuclear region (orange; merge). WND (green) and MNK (red) showed a perinuclear and cytoplasmic distribution, with no significant co-localization of the two proteins in the perinuclear region of the Jeg-3 cells (Figure 1E).

Effect of hormone treatment on MNK and WND protein expression using Western blot analysis

The effect of hormone treatment for 3 days on the expression of MNK in Jeg-3 cells grown on porous membranes was determined using Western blot analysis (Figure 2). The hormone concentrations used are similar to the levels present in the maternal circulation during the third trimester. These results are representative of protein extracts from three independent hormone treatments, each conducted in triplicate. The protein levels of MNK were determined relative to β -actin by densitometry. Treatment with 10 ng/ml insulin (Figure 2A, lane 2) resulted in a 3.1-fold (± 0.3) increase of MNK (Figure 2A, compare lane 1

with lane 2; $P < 0.001$). There was a 2.3-fold (± 1.5) increase in the levels of MNK in the cells treated with 2.7 ng/ml oestrogen (Figure 2A, lane 5; $P < 0.001$). Cells treated with 180 ng/ml IGF-I (Figure 2A, lane 3), 1100 ng/ml IGF-II (Figure 2A, lane 4) or 0.015 ng/ml progesterone (Figure 2A, lane 6) showed no significant change in the levels of the MNK protein, relative to the untreated cells. No effects of insulin, IGF-I, IGF-II, oestrogen or progesterone were detected after 1 h, 3 h or 24 h treatments (results not shown).

To investigate the hormone response of MNK further, Jeg-3 cells were treated with increasing concentrations of insulin or oestrogen for 3 days (Figures 2B and 2C respectively). These results are representative of protein extracts from three independent hormone treatments, each conducted in triplicate. The two lower concentrations of insulin (2.5 and 5 ng/ml) did not result in any change in MNK levels, but in agreement with Figure 2(A), a significant increase of 4.1-fold (± 0.3) and 5.4-fold (± 0.7) was observed in cells treated with 10 ng/ml insulin (Figure 2B, lane 4) and 20 ng/ml insulin (Figure 2B, lane 5) ($P < 0.001$). Similarly, the protein levels of MNK in extracts from cells treated with oestrogen also increased in response to increasing oestrogen levels, showing a 1.5-fold (± 0.17), a 1.7-fold (± 0.06), a 2.1-fold (± 0.19) and a 3.5-fold (± 1.2) increase when treated with 0.7 ng/ml (Figure 2C, lane 2), 1.35 ng/ml (Figure 2C, lane 3), 2.7 ng/ml (Figure 2C, lane 4) and 5.4 ng/ml (Figure 2C, lane 5) respectively.

The effect of 3 day hormone treatment on the expression of WND in Jeg-3 cells grown on porous membranes was determined using Western blot analysis (Figure 3). Treatment with 10 ng/ml insulin produced a reduction of WND to 0.06 ± 0.03 ($P < 0.001$) of the level of the untreated cells (Figure 3A, lane 2 and lane 1 respectively). In contrast, cells treated with IGF-I, IGF-II, oestrogen or progesterone resulted in no significant change in the levels of the WND protein. No effect on WND expression was detected when treated with insulin, IGF-I, IGF-II, oestrogen or progesterone for shorter time intervals of 1 h, 3 h or 24 h treatments (results not shown).

Cells were treated for 3 days with different concentrations of insulin to determine the effect on WND expression levels (Figure 3B). Three independent hormone treatments were used for each point and each sample was analysed on three Western blots. The protein levels of WND were unaltered in the presence of 2.5 ng/ml insulin, but decreased to 0.6 of control levels in 5 ng/ml insulin (Figure 3B, lane 3; $P < 0.001$). Similar to the results in Figure 3(A), 10 ng/ml insulin (Figure 3B, lane 4) caused a reduction to 0.15 ± 0.05 of control levels; however, the higher dose of 20 ng/ml insulin (Figure 3B, lane 5) caused a less marked reduction to 0.7 ± 0.5 of control levels ($P < 0.001$).

Effect of hormone treatments on MNK and WND mRNA levels in Jeg-3 cells

MNK mRNA levels, measured by real-time PCR, were increased by 4.71-fold (± 0.32) in cells treated with 10 ng/ml insulin for 3 days ($P < 0.001$; Figure 4A). An increase of 3.73-fold (± 0.31) was found in cells treated with 2.7 ng/ml oestrogen ($P < 0.001$). The increased levels of *MNK* mRNA in hormone-treated cells were similar to the increases in protein detected by Western blot analyses (Figure 2). The mRNA levels of *WND* were not significantly reduced following insulin and oestrogen treatment for 3 days relative to the untreated cells (Figure 4B; 0.73 ± 0.3 , 0.77 ± 0.23 and 1.02 ± 0.29 respectively). Hormone treatments were also conducted over 1 h, 3 h and 24 h time periods and no significant change in the mRNA of either *MNK* or *WND* when treated with insulin, IGF-I, IGF-II, oestrogen or progesterone was

Figure 1 Detection and immunolocalization of MNK and WND protein in Jeg-3 cells

Total protein was isolated from Jeg-3 cells and separated by SDS/PAGE [7.5 % (w/v) polyacrylamide]. Western blot analysis of MNK protein (**A**) and WND protein (**B**) in Jeg-3 cells (Lane 2). Similar sized bands were also found in PMC42 breast cell extracts, which were used as a positive control (Lane 1). Double label indirect immunofluorescence of MNK (green) and p230 TGN marker (red) (**C**), WND (green) and TGN marker (red) (**D**) and WND (green) and MNK (red) (**E**) in Jeg-3 cells. Bar = 10 μ m.

observed (results not shown). All PCR reactions (1 h, 3 h, 24 h and 3 days) were performed using RNA extracted from cells from three independent hormone treatments, and each reaction was conducted in triplicate.

Effect of hormones on the localization of MNK and WND in Jeg-3 cells

Immunofluorescence showed that in both untreated cells and cells treated with 10 ng/ml insulin for 24 h, MNK had a perinuclear distribution as well as a diffuse cytoplasmic label (Figures 5A and

5B respectively). The z-section shows perinuclear label in the untreated cells. In the insulin-treated cells, however, there was a greater proportion of diffuse cytoplasmic label with less perinuclear label compared with the control cells. The z-section also shows that in the insulin-treated cells MNK has a more diffuse labelling, including some basolateral label (Figure 5B), relative to the untreated cells. In the cells treated with 2.7 ng/ml oestrogen for 24 h, MNK had a diffuse cytoplasmic, punctate distribution (Figure 5D). This is confirmed by the cytoplasmic label in the z-section which also shows some basolateral labelling of MNK. This is in contrast with the untreated cells, where MNK had a

Figure 2 Effect of hormone treatment on MNK protein expression

Total protein was isolated from hormone-treated Jeg-3 cells and separated by SDS/PAGE [7.5 % (w/v) polyacrylamide]. **(A)** Untreated Jeg-3 cells (Lane 1) and Jeg-3 cells treated with 10 ng/ml insulin (Lane 2), 180 ng/ml IGF-I (Lane 3), 1100 ng/ml IGF-II (Lane 4), 2.7 ng/ml oestrogen (Lane 5) or 0.015 ng/ml progesterone (Lane 6). **(B)** Untreated Jeg-3 cells (Lane 1) and Jeg-3 cells treated with insulin at 2.5 ng/ml (Lane 2), 5 ng/ml (Lane 3), 10 ng/ml (Lane 4), or 20 ng/ml (Lane 5). **(C)** Untreated Jeg-3 cells (Lane 1) and Jeg-3 cells treated with oestrogen at 0.7 ng/ml (Lane 2), 1.35 ng/ml (Lane 3), 2.7 ng/ml (Lane 4) or 5.4 ng/ml (Lane 5). The membranes were re-probed with an antibody to the housekeeping protein β -actin indicating equal loading of sample protein from each cell extract.

Figure 3 Effect of hormone treatment on WND protein expression

Total protein was isolated from hormone-treated Jeg-3 cells and separated by SDS/PAGE [7.5 % (w/v) polyacrylamide]. **(A)** Untreated Jeg-3 cells (Lane 1) and Jeg-3 cells treated with 10 ng/ml insulin (Lane 2), 180 ng/ml IGF-I (Lane 3), 1100 ng/ml IGF-II (Lane 4), 2.7 ng/ml oestrogen (Lane 5) or 0.015 ng/ml progesterone (Lane 6). **(B)** Untreated Jeg-3 cells (Lane 1) and Jeg-3 cells treated with insulin at 2.5 ng/ml (Lane 2), 5 ng/ml (Lane 3), 10 ng/ml (Lane 4) or 20 ng/ml (Lane 5). The membranes were re-probed with an antibody to the housekeeping protein β -actin indicating equal loading of sample protein from each cell extract.

Figure 4 Effect of hormone treatments on MNK and WND mRNA levels in Jeg-3 cells

RNA was extracted from hormone-treated Jeg-3 cells and the relative mRNA level determined by real-time PCR with GAPDH as the internal control. Bar graphs of cells treated for 3 days with either insulin or oestrogen and compared with untreated cells. **(A)** MNK and **(B)** WND. Asterisks denote a significant difference ($P < 0.001$).

predominantly perinuclear localization, with some cytoplasmic labelling (Figure 5C). In the insulin-treated cells, WND had a perinuclear distribution, with little general cytoplasmic labelling compared with the untreated cells, which had a diffuse cytoplasmic label (Figures 5F and 5E respectively). This localization is confirmed in the z-section. Oestrogen treatment had no effect on the localization of WND in the Jeg-3 cells (results not shown).

Effect of BCS and hormones on the localization of MNK and WND in Jeg-3 cells

The changes in localization of MNK and WND in response to insulin and oestrogen could be due to a direct effect of the hormones on the trafficking of the protein or be occurring as a secondary response to an increase in cytoplasmic copper induced by the hormone treatment. To distinguish between these possibilities, Jeg-3 cells were treated with 100 μ M of the copper chelator BCS for 24 h prior to, and during, the hormone treatment. In Jeg-3 cells treated with 100 μ M BCS for 24 h, MNK had a predominately perinuclear distribution, which is confirmed by the z-section, where MNK was adjacent to the nuclei (Figures 5G and 5I). When these cells were treated with 10 ng/ml insulin for a further 24 h in the presence of 100 μ M BCS, the redistribution of MNK from the perinuclear region to a diffuse cytoplasmic pattern still occurred (Figure 5H). Similarly, when the Jeg-3 cells were treated with 2.7 ng/ml oestrogen for 24 h, after the BCS treatment, MNK also had a diffuse cytoplasmic label, relative to the BCS-treated cells (Figure 5J). WND remained in a perinuclear location following treatment of cells with 10 ng/ml insulin, in the presence or absence of 100 μ M BCS (Figures 5L and 5K respectively). These experiments suggest that insulin has an effect on the localization

Figure 5 Effect of hormones and BCS on the localization of MNK and WND in Jeg-3 cells

(A–F) Indirect immunofluorescence of MNK and WND in Jeg-3 cells treated with hormones for 24 h. MNK (green) in untreated cells (A and C), cells treated with insulin (B) and oestrogen (D). WND (green) in untreated cells (E) and cells treated with insulin (F). XY-sections shown with Z-section below. Nuclei labelled with ethidium bromide (red). Bar = 15 μ m. (G–L) Indirect immunofluorescence of MNK and WND in Jeg-3 cells treated with 100 μ M BCS and hormones for 24 h. MNK (green) in BCS-treated control cells (G and I), cells treated with BCS and insulin (H) and BCS and oestrogen (J). WND (green) in BCS-treated control cells (K) and cells treated with BCS and insulin (L). XY-sections shown with Z-section below. Nuclei labelled with ethidium bromide (red). Bar = 20 μ m.

of MNK and WND that is not mediated through changes in the copper concentration in the cell.

Effects of hormones on the transport and accumulation of copper in Jeg-3 cells

To investigate whether the hormonally induced changes in MNK and WND affected copper transport, cells were grown on EHS-matrix coated PET membranes and vectorial transport of ^{64}Cu was determined as described in the Experimental section. Confluent Jeg-3 cells were treated with either insulin (10 ng/ml) or oestrogen (2.7 ng/ml) for 3 days and the transport of copper from the apical to basolateral chamber and vice versa was determined (Figure 6). Copper transport from the apical to the basolateral surface over a 6 h time period, was significantly increased at 2 h, 4 h and 6 h in Jeg-3 cells treated with insulin relative to the control cells (Figure 6A; $P < 0.01$). In addition, the transport of copper was also increased at 4 h and 6 h in Jeg-3 cells treated with oestrogen (Figure 6A; $P < 0.01$). In contrast, the transport of copper from the basolateral surface to the apical surface was significantly decreased in Jeg-3 cells treated with either insulin or oestrogen at 4 h and 6 h time points, when compared with the untreated Jeg-3 cells (Figure 6C; $P < 0.001$).

The effects of hormones on the intracellular levels of copper were determined following addition of radioactive copper to either the apical or basolateral chamber. In both situations there was a significant decrease in the intracellular levels of copper in Jeg-3 cells treated with insulin or oestrogen, when compared with the untreated Jeg-3 cells (Figures 6B and 6D respectively; $P < 0.01$), suggesting that the cells had an enhanced ability to efflux copper. These results are representative of three independent

^{64}Cu transport and accumulation assays, each conducted in duplicate.

MNK promoter region, IRSs and EREs

The response of *MNK* mRNA to insulin may be due to increased transcription and, if so, the promoter region of MNK would contain consensus sequences for IRSs and EREs. Using ClustalX® software it was determined that the MNK promoter may contain an IRS located between –1421 and –1428 (Figure 7A), where it shows complete alignment with the IRS CAAATAA [24]. For the ERE, the consensus sequence is an inverted repeat of the sequence GGTCA separated by three base-pairs [25]. Slight variations of the EREs which differ from the inverted repeat consensus sequence by one or two base-pairs are considered as alternative binding sites [26]. In the present study it was found that the MNK promoter region also has a possible ERE, where the GGTCA sequence is mismatched at 2 base-pairs. The ERE sequence is AGGTCTNN/TGACCT and an alignment with this sequence is located between –1845 and –1859, and differs as follows; ACCTTACAAGGACCT (Figure 7B). No IRS or ERE was located in the promoter region of WND (results not shown). Further work is required to establish whether the mRNA responses are due to transcriptional regulation.

DISCUSSION

The present study has shown that both MNK and WND were present in the polarized Jeg-3 cells, where they had a similar perinuclear localization but also were differentially localized

Figure 6 Effect of hormones on the transport and accumulation of ^{64}Cu in Jeg-3 cells

Transport and accumulation of ^{64}Cu in Jeg-3 cells treated with insulin or oestrogen for 3 days. A line and bar graph of the pmol of $\text{Cu}/\mu\text{g}$ of protein as measured from the apical surface to the basolateral surface, per h for 6 h, in control, insulin-treated or oestrogen-treated Jeg-3 cells (A and B). A line and bar graph of the pmol of $\text{Cu}/\mu\text{g}$ of protein as measured from the basolateral surface to the apical surface, per h for 6 h, in control, insulin-treated or oestrogen-treated Jeg-3 cells (C and D). Asterisk denotes a significant difference ($P < 0.001$).

throughout the cytoplasm. This is similar to the localization of these proteins in the trophoblast cells seen in our previous analysis of human placental tissue [15]. The overlap of both MNK and WND with a TGN marker suggests that the proteins were located in this perinuclear region. These observations are consistent with previous studies, which have shown that MNK and WND are localized within the TGN in CHO (Chinese-hamster ovary) cells and HepG2 cells [27–29], where they supply copper to copper-dependent enzymes and ceruloplasmin respectively. The TGN localization of the MNK and WND proteins in the Jeg-3 cells may indicate a similar function for MNK and WND in trophoblasts.

The increase in MNK mRNA and protein levels in response to treatment with insulin and oestrogen is a novel finding, as no

previous data on hormone regulation of this ATPase has been reported. Insulin regulates metabolism by altering the synthesis, stability or translation of many mRNAs including GLUT3, Ca^{2+} -ATPase and Na^{+} - K^{+} -ATPase often via IRSs [30]. Although we have not established whether insulin and oestrogen directly enhanced MNK transcription, we have identified a likely IRS and ERE in the promoter sequence of MNK. The effects of insulin and oestrogen on the MNK protein and mRNA levels were only evident at 72 h of hormone treatment. In some systems the effect of insulin on gene expression is rapid, between 30 min to 12 h, as seen in the insulin-promoted increase of the GLUT1 mRNA in the first trimester human trophoblast-like cells [16,31]. However, other studies have shown that the insulin-induced transcription of other proteins including albumin, casein and the pancreatic amylase gene is slow in onset and occurs after 24 h of treatment [30]. An insulin-induced increase in leptin mRNA in human trophoblast cells occurred only after 72 h treatment with insulin [32], and so the response time of MNK to insulin is in line with this group of slower responding genes.

Insulin and oestrogen also changed the localization of MNK in the Jeg-3 cells, causing MNK to become more diffusely distributed in the cytoplasm with some accumulation at the basolateral membrane relative to the untreated cells. This localization pattern is consistent with the basolateral localization of MNK we observed in the syncytiotrophoblast layer of human placental tissue [15]. This relocation together with the increased levels of expression of MNK will presumably increase copper transport to the foetus. This supposition is supported by the copper transport assay data, where treatment with insulin and oestrogen increased the transport of copper across the basolateral membrane in Jeg-3 cells.

The present paper is the first report to show that hormones cause trafficking of these copper-ATPases. Previous studies have demonstrated that exposure to high copper concentrations induces MNK to traffic to the plasma membrane and WND to move from the TGN to large vesicles [27,28]. Thus it is possible that the hormone-induced trafficking was due to stimulation of copper uptake, resulting in increased cytoplasmic copper leading to trafficking of the ATPases. This possibility was eliminated by demonstrating that trafficking in response to the hormones still occurred in the presence of the copper chelator BCS. The only other case of trafficking of MNK unrelated to changes in copper concentration is the activation of glutamate receptors in primary hippocampal neurons that result in increased efflux of copper. The mechanism of copper-induced trafficking of WND and MNK is still not clarified, but there is some evidence that changes in phosphorylation are involved [33,34]. Treatment with oestrogen and insulin may cause similar phosphorylation changes leading to the trafficking of the proteins.

The increase in MNK levels and the changes in localization in response to insulin and oestrogen may be related to the needs of the growing foetus for copper. As gestation advances, the secretion of insulin from the pancreas is three times higher than in non-pregnant women [35]. The levels of oestrogen both secreted from the placenta and within the maternal circulation increase after the first trimester through to term [36]. If these elevations of insulin and oestrogen result in an increase of MNK in the placenta similar to that observed in the Jeg-3 cells, we would predict that the capacity of the placenta to supply copper to the foetus across gestation would be substantially enhanced. Presumably as the foetus grows, the amount of copper crossing the placenta to supply the copper-dependent enzymes in the foetus must increase. Moreover, during the later stages of pregnancy, copper is stored in the liver of the foetus as a source of copper for the neonate [37] also increasing the amount of copper crossing the placenta.

A **B** **Figure 7** Alignment of the MNK promoter region and IRS or ERE

Alignment of promoter region of MNK with the IRS (**A**), and the ERE (**B**). Asterisk denotes complete base pair alignment.

Figure 8 Cell model of insulin and oestrogen effect on MNK and WND in the syncytiotrophoblast layer of human placenta

Illustration of the predicted effect of insulin and oestrogen on MNK and WND in trophoblast placental cells. Increased insulin and oestrogen levels result in MNK trafficking to the basolateral membrane, with increased Cu efflux towards the foetal circulation. In contrast, WND relocates to the TGN, in response to increased insulin levels, to decrease the efflux of Cu towards the maternal circulation.

The present study shows evidence that the expression and localization of the WND protein is also influenced by insulin. The expression was reduced by as much as 10-fold in the insulin-

treated Jeg-3 cells, but surprisingly with no change in the corresponding mRNA. Insulin may therefore regulate the levels of WND by decreasing the stability of the protein or reducing translational efficiency of the mRNA, rather than altering the synthesis or transcription of *WND*. This is similar to the regulation of GLUT4 by insulin. It has been shown in adipocytes that insulin represses the levels of the GLUT4 mRNA and protein; however, no IRS has been identified in the GLUT4 promoter [38]. It has been speculated that insulin may mediate its negative effect on GLUT4 by inhibiting the transcription of the β 3-adrenergic receptor and thereby have an indirect effect on the cAMP pathway [39]. This indirect effect of insulin on protein expression and consequent function may be similar to the effect of insulin in decreasing WND in the placental Jeg-3 cells.

As well as reducing WND levels, insulin also changed the intracellular localization of the protein. In the insulin-treated cells, WND was found in a tight perinuclear region, rather than the more diffuse distribution found in untreated cells. The reduction in amount and the restriction of the protein to the TGN region, suggests that insulin is acting to reduce the copper efflux role of WND, which would normally occur across the apical surface of the placental trophoblast, i.e. into the maternal circulation.

Our data is consistent with a model of placental transport of copper in which MNK and WND have complementary roles (see Figure 8). We propose that MNK has a primary role in copper delivery to the foetus across the basolateral surface of the syncytiotrophoblast layer. This role is supported by the present study and previous work that demonstrated failure of placental transport of copper in mouse models of Menkes disease [12] and the accumulation of copper in the placentas from babies with Menkes disease [40]. The increase in expression of MNK in response to insulin and oestrogen and the changes of localization of the protein

act to increase the transport of copper across the basolateral membrane and would result in increased copper supplies to the foetus as pregnancy proceeds. We also suggest that WND has a protective role in early pregnancy, ensuring that excess copper is returned to the maternal circulation across the apical surface of the syncytiotrophoblast layer, but that this role is less important, and indeed may be detrimental, in the latter stages of pregnancy, when an increase in foetal copper is in demand.

In summary, it was found that both MNK and WND are expressed in the Jeg-3 choriocarcinoma cell line, both have perinuclear localization and are differentially localized in the cytoplasm of these cells. It was determined that insulin increases both the RNA and protein levels of MNK and the identification of the IRS in the promoter sequence of MNK suggests that this effect may be transcriptional. Insulin changed the localization of MNK from the perinuclear region and cytoplasmic distribution to the basolateral membrane and increased the transport of copper towards the basolateral membrane of Jeg-3 cells. In addition, oestrogen also altered the levels of MNK by increasing both the RNA and protein levels of the ATPase via a possible ERE located within the promoter region of MNK. In contrast to MNK, insulin decreased the protein levels of WND, directed the protein to the perinuclear region and decreased the efflux of copper at the apical membrane of Jeg-3 cells, all of which may be consistent with decreased copper transport towards the maternal circulation within placental tissue. These data are consistent with a model that proposes differential regulation of MNK and WND during pregnancy to ensure safe but adequate copper supplies to the developing foetus.

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