Placental biology of Down syndrome in relation to increased gene dosage

OGW Wong, HYS Ngan, ANY Cheung *

KEY MESSAGES

1. Amyloid precursor protein (APP) is overexpressed in the placentas of Down syndrome subjects.
2. Inducible APP overexpressed trophoblast cell line models are established.
3. APP overexpression dysregulates trophoblast cell functions.

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[1] **OGW Wong,** [2] **HYS Ngan,** [1] **ANY Cheung**

Queen Mary Hospital, The University of Hong Kong:
[1] *Department of Pathology*
[2] *Department of Obstetrics and Gynaecology*

* Principal applicant and corresponding author: anycheun@pathology.hku.hk

Introduction

Down syndrome (DS) is the most common congenital abnormality in humans. Trisomy 21 is the major cause and accounts for about 95% of DS. DS manifests a spectrum of phenotypes, including retardation in cognitive ability and physical growth, cardiac defects, craniofacial alterations, and muscle hypotonia.[1] Among women diagnosed to have DS fetus by prenatal screening, the induced abortion rate is estimated to be 50% to 90%. A reduction in the termination rate is observed in recent years, and hence there may be an increasing number of DS individuals whose medical conditions need to be taken care of. Functional characterisation of genes dysregulated in DS may facilitate development of potential gene therapy strategies to correct phenotypic abnormalities in DS.

Defects in DS placentas have been well documented.[2] Histologically, more conspicuous two-layered trophoblast in the chorionic villi of trisomy 21 placentas is noted secondary to increased presence of mononuclear cytotrophoblast. Cultured villous cytotrophoblast cells from trisomy 21 aggregate normally but fuse inefficiently to form multinucleated syncytiotrophoblast. DS placenta also exhibits dysregulated hCG physiology. The number of mature hCG receptor (LH/CG-R) molecules expressed on the surface of trisomy 21-affected cytotrophoblasts is significantly reduced. Nonetheless, the molecular basis underlying the alteration in trophoblast differentiation and hCG signalling in DS trisomy 21 placentas are largely unknown.

Various genetic mechanisms of DS have been suggested. For instance, critical regions in chromosome 21 may account for pathogenesis of DS. Various phenotypes of DS are considered to be results of the extra copy of dosage sensitive genes among the genes present on human chromosome 21 (the 'gene dosage' hypothesis). A list of genes with direct evidence of increased dose or allelic variation that may induce one or more phenotypes of DS has been compiled.[3] Placental dysfunction is associated with intrauterine growth retardation, hypertension, hypoxic-ischaemic injury, preterm labour, and fetal death. Trophoblast cell biology plays important roles in these diseases. For example, aberrant cell death signals are associated with increased p53 activity and altered translation of AKT, and mTOR proteins are crucial in pathogenesis of intrauterine growth retardation.[4] Moreover, almost all DS individuals suffered from stunted growth at birth and in adulthood. Therefore, it is important to include a placental trophoblast model in the study of DS.

This study aimed to (1) investigate the expression profiles of amyloid precursor protein (*APP*), *ETS2*, *SOD1*, and *HMGN1*, in trisomy 21 placentas, (2) establish inducible overexpression trophoblast cell line models, (3) investigate the phenotypic (proliferation, apoptosis, invasion, hCG secretion, LH/CG-R expression, and differentiation) changes of trophoblast upon overexpression of dosage sensitive genes, and (4) delineate the molecular mechanisms of dosage sensitive genes with particular focus on the hCG-PI3K-Pak4 and Nanog signalling pathways known to affect trophoblast pathology.

Methods

A total of 71 formalin-fixed paraffin-embedded placental tissues, including 37 placentas from normal pregnancies and 34 placentas from DS pregnancies were retrieved from the archive of Department of Pathology, Queen Mary Hospital. The use of the samples was approved by the institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (ref. UW 13-124).

Five 10-μm sections were cut from each placenta and the total RNA was extracted using the RNeasy FFPE kit (Qiagen). At least 500 ng total RNA was reverse-transcribed into cDNA using the PrimeScript RT kit (Clontech). Each RT-qPCR reaction contains 1X HotStart SYBR Green qPCR master mix (Excell), 1 μM forward primer, 1 μM reverse primer, 0.5 μL cDNA, and 4.5 μL milli-Q water. RT-qPCR reactions were run using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Immunohistochemical study was performed with antigen retrieval through heating under pressure. The sections were then incubated with anti-APP antibody (ab32136, Abcam) at room temperature overnight and the signal was visualised using the EnVision+ Dual Link System. The stained sections were scanned with the Aperio slide scanner, and the staining intensity was evaluated by the positive pixel count v9 algorithm provided by the system.

Western blot was performed according to standard procedures. Antibodies used included: APP (ab32136, Abcam), GFP (ZsGreen, 632598, clontech), β-actin (A5060, Sigma-Aldrich), Caspase 3 (#9662, Cell Signaling), LHCGR (19968-1-AP , Proteintech), PAK4 (#3242, Cell Signaling), p-PAK4 (#3241, Cell Signaling), NANOG (14295-1-AP, Proteintech), p-PI3K p85 (#4228, Cell Signaling), PI3K p85 (#4257, Cell Signaling), and Akt (#9272, Cell Signaling).

The functional implication of APP overexpression was investigated in two trophoblast cell line HTR-8/SVneo.[5] HTR-8/SVneo was stably transfected with a Tet-On expression plasmid pTRE3G-BI-APP and yielded two clones B2 and B10. APP was induced in B2 and B10 by including the recombinant protein Tet-Express (Clontech) in the culture medium.

Cell growth and cell proliferation was measured by the MTT assay and BrdU assay, respectively. Cellular senescence was detected using senescence associated β-gal assay. Cell cycle analysis was performed by flow cytometry. Cell migration and invasion activities were measured by transwell migration/invasion assay.

Results

APP is overexpressed in the placentas of Down syndrome

Four candidate genes: *SOD1*, *ETS2*, *APP*, and *HMGN1* located on chr21 were selected for expression characterisation in DS placentas using RT-qPCR. *YWHAZ*, a gene previously demonstrated to be a good reference gene for gene expression study in placenta,[6] was used as the reference gene. We observed that APP was significantly overexpressed in DS than normal placentas (mean, 2.498-fold; P=0.0009). In addition, *HMGN1* was markedly suppressed in DS than normal placentas (mean, 0.2714-fold; P<0.0001). *ETS2* and *SOD1* did not show obvious change in expression between DS and normal placentas. Two additional reference genes *GAPDH* and *TOP1* were used for calculation and yielded similar observations.

The overexpression of APP in DS placenta was verified by immunohistochemistry. Cytoplasmic immunoreactivity was found with focal membranous accentuation. The immunoreactivity was present at cytotrophoblast, syncytiotrophoblast, extravillous implantation site trophoblast, and villous stromal cells. There was a significant increase in APP protein expression in DS than normal placentas (P<0.0001) [Fig].

APP overexpression suppressed growth and invasiveness of trophoblast

Growth curves constructed by the MTT assay suggested that APP induction mildly slowed cell growth. This effect was not due to reduced cell proliferation upon APP overexpression, as BrdU incorporation rate did not show significant difference in cells with or without APP induction. Instead, there was an increase in the number of apoptotic cells as in APP-induced B2 and B10 cells as evidenced by flow cytometry analysis of DNA fragmentation, TUNEL assay, and caspase-3 cleavage assay. B2 and B10 cells migrated and invaded slower through transwells when APP was induced.

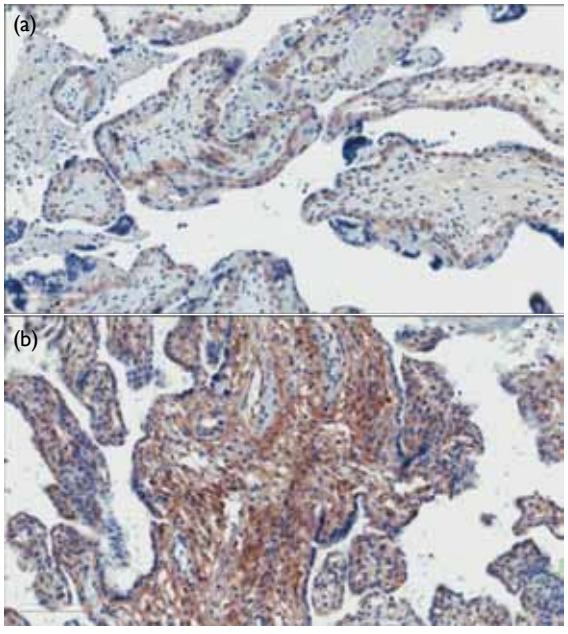

FIG. Cellular expression pattern of amyloid precursor protein in (a) normal and (b) Down syndrome placentas.

APP induction dysregulated hCG level

Reduced alpha and beta hCG mRNA was observed in APP-induced B2 and B10 cells in association with reduced syncytialisation. However, no change in LH/CG-R, PAK4, p-PAK4, or NANOG protein expression could be detected upon APP induction. Expression level and phosphorylation status of PI3Kwere not affected by APP.

APP was confirmed to be overexpressed in DS placentas in association with dysregulation of trophoblast cell functions. This may contribute to the abnormal phenotypes observed in DS placentas.

Discussion

APP is the very first protein known to be upregulated in the brain of DS individuals.[7] APP is associated with mental underdevelopment, and DS individuals frequently develop Alzheimer disease at young age. APP is implicated in the abnormalities seen in other organs of DS subjects as well. We confirmed that APP is overexpressed in the placentas of DS subjects. The mRNA expression was accessed by RT-qPCR, and the upregulation was confirmed by immunohistochemistry.

We then investigated the pathological function of APP in DS placentas by establishing inducible expression systems in two trophoblast models HTR-8/SVneo. Doses of inducer APP were manipulated so that the induced protein expression level in HTR-8/SVneo was comparable to what is observed in the clinical samples. APP overexpression in HTR-8/SVneo was found to induce reduction of cell growth in relation to increased apoptosis as evidenced by flow cytometry, TUNEL, and caspase-3 cleavage assays. APP has been shown to participate in apoptosis induction in olfactory neurons.[8] Cytotrophoblast differentiation into syncytiotrophoblast was suppressed as evidenced by reduced hCG production and syncytialisation. Upon APP induction, reduced trophoblast motility was also observed. Previous characterisation of DS trophoblast has revealed that these cells failed to properly switch their expression of stage-specific antigens towards an invasive phenotype.[9] Invasive ability of trophoblasts at decidua and maternal blood vessels play critical role in various functions of the placenta.[10]

Conclusion

Dysregulation of trophoblast cell functions upon APP induction may play a role in abnormal placental development in DS individuals.

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