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The untapped potential of placenta-enriched molecules for diagnostic and therapeutic development

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A B S T R A C T

Pregnancy complications such as fetal growth restriction and preeclampsia are diseases with limited biomarkers for prediction, and a complete lack of therapeutic options. We define placenta-enriched molecules as those that are highly expressed in the placenta relative to all other human tissues. Many exist including mRNAs, miRNAs and proteins. It is now well established that placenta-enriched mRNAs are found within the maternal circulation and are cleared rapidly after birth. Similarly, distinct clusters of miRNAs that are placenta-enriched have been identified and are measurable within the circulation. However, perhaps the most established potential diagnostics thus far are circulating placental proteins such as placental growth factor (PlGF), pregnancy associated pregnancy protein-A (PAPP-A) and soluble FMS-like tyrosine kinase 1 (sFlt-1). There has also been much interest in targeting placenta-enriched molecules as a means to treat diseases of pregnancy. We have shown promising results in targeting placenta-enriched epidermal growth factor receptor (EGFR) to treat ectopic pregnancy. Others have focused on using placenta-enriched molecules as a means of homing therapeutic-filled nanoparticles to the placenta, or to directly target sFlt-1 to improve disease outcomes. Importantly, many placenta-enriched molecules remain largely unstudied. We propose that a better understanding of their biology, and potential contribution to the pathogenesis of diseases, may yield more predictive diagnostic and therapeutic targets.

1. Introduction

The placenta is the life-blood of the fetus, providing the vascular interface for the nutrients and oxygen required to sustain it throughout gestation. The only temporary adult organ, the placenta is of fetal origin, and is essentially foreign to the mother. It is therefore unsurprising that it has its own specific transciptome and proteome. A set of molecules exist that are exclusively or highly expressed in the placenta relative to all other human cells and tissues. Defined hereon as "placenta-enriched molecules", many exist (and potentially more remain to be identified). While some placenta-enriched molecules already have well-defined roles in disease, for some little to no study has been undertaken. In this review, we highlight the placenta-enriched molecules that are currently and commonly being used as diagnostic biomarkers. We also outline the potential of placenta-enriched molecules as targets for therapeutics; and in better understanding diseases of pregnancy including preeclampsia and fetal growth restriction.

2. Placenta-enriched genes as biomarkers

The placental transcriptome includes placenta-enriched mRNAs and microRNAs. Dennis Lo and others have led the way in demonstrating that placental mRNA transcripts are readily detectable within the maternal circulation. They have shown that some may correlate with the circulating proteins they encode, and that they are cleared rapidly after birth [1]. Our team have an interest in measuring placenta-enriched circulating mRNAs as potential biomarkers for pregnancy disorders. Using *in silico* analysis, we previously identified 137 mRNA transcripts that are highly expressed in the placenta relative to other human tissues [2]. We have subsequently measured many of these within the circulation and shown some to be differentially expressed in a number of conditions which represent placental dysfunction. For example, in preeclampsia we have shown that PLAC3, PLAC4, CRH and ERVWE1 [3] are differentially expressed in the circulation (whole blood) of women with established preeclampsia (average gestation at sampling was 31.1 weeks) relative to gestation matched samples from women who delivered healthy babies at term. These may have potential as biomarkers for the disease. In a separate study, we investigated placenta-enriched mRNA transcripts and hypoxia-regulated mRNAs in the maternal circulation throughout labour to determine if the fetus was hypoxic in utero. We found that several mRNAs including placenta-enriched Adrenomedullin correlated with increasing fetal hypoxia as

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labour progressed [4]. In the same report we showed that these mRNAs were also differentially expressed in the maternal circulation of fetuses affected by severe preterm growth restriction. The expression of these mRNAs correlated with Doppler velocimetry abnormalities in fetal vessels reflecting chronic hypoxia [4].

MicroRNAs (miRNAs) are small non-coding RNA molecules and are important in the regulation of gene expression [5]. miRs also form part of the placental transcriptome. Up to 42% of miRs in the human genome exist in clusters [6]. Several of these clusters are primarily expressed in the placenta or are placenta-specific. Two examples of such clusters are the C19MC and C14MC clusters. The C19MC cluster is inherited in the placenta from the paternal allele and is primate specific [7]. The C14MC cluster is inherited from maternally imprinted genes [8]. Circulating levels of miRNAs from the C19MC cluster increase from first to third trimester. In contrast, those from the C14MC cluster decrease over gestation, indicating that the different clusters have important roles at different stages of pregnancy [9].

Several miRNAs in primary trophoblast cells are regulated by hypoxia [10,11]. Some of these 'hypoxamiRS' have been shown to be upregulated in the maternal circulation throughout labour [12] as the fetus experiences progressive labour-induced hypoxia including miR 210, miR 21, miR 424, miR 199a, miR 20b, and miR 373. This suggests that, measurement of circulating miRs may offer a snapshot assessment of fetal health.

Overall, the placenta is a rich source of measurable mRNAs and miRNAs that are readily released into the maternal circulation. Reflecting the functional transcriptome of the placenta, measurement of circulating placenta-enriched mRNAs and miRNAs offers a unique opportunity to obtain a non-invasive window into the uterus. This could potentially be used to assess the health of both the placenta and the fetus. For further detail regarding circulating nucleic acids as biomarkers, we refer readers to a review by Whitehead et al. [13]. This review outlines the potential of non-invasively profiling the placental transcriptome via a maternal blood sample.

3. Placenta-enriched proteins as biomarkers

Whilst the detection of circulating mRNAs and miRNAs is a reasonably recent emerging field, placenta-enriched proteins have been studied for many years.

Human chorionic gonadotrophin (hCG) is the most commonly used placental biomarker. It is one of the earliest markers produced by the conceptus and placenta, detectable as early as two weeks after implantation. As such, it is commonly used to determine pregnancy status via urine and blood tests [14].

Some screening tests for Trisomy 21 (Down's syndrome) use circulating protein concentrations, along with ultrasound markers and/or maternal characteristics, to calculate the risk for that pregnancy [15]. The same proteins that are used to screen for Down Syndrome have consistently been associated with cases of small-for-gestational-age (SGA; < 10th centile) infants. To date, pregnancy-associated plasma protein A (PAPP-A) has appeared to be the most reliable [16–19].

PAPP-A is a well-known placenta-enriched molecule, almost exclusively expressed by the placenta. It originates from trophoblast cells and binds insulin-like growth factors [20]. It is directly involved in placental function and fetal growth [21]. Low first-trimester PAPP-A multiples of the median (< 0.4MoM/ < 5th centile) has been consistently associated with infants later born SGA, with an odds ratio of 2.5–2.9 [16,22]. This odds ratio qualifies low PAPP-A as a major risk factor for SGA according to the Royal College of Obstetricians and Gynaecologists. It is therefore recommended that women with first trimester PAPP-A < 0.4 MoM have serial growth ultrasound scans in the third trimester. The aim of this is to increase the identification of SGA fetuses in order to institute surveillance and timely delivery to prevent stillbirth. This demonstrates the important clinical role placenta-enriched proteins can play. Using the same cut-off (0.4MoM), PAPP-A has also been shown to have a sensitivity of 39% for predicting early onset preeclampsia, with a specificity of 87% [16].

hCG is the other important placenta-enriched protein used in first trimester screening. hCG concentrations have also been associated with predicting SGA infants, not only in first trimester screening but also in second trimester screening. The association with SGA however is far weaker than that of PAPP-A. hCG is secreted by syncytiotrophoblasts. It is vital in signalling the corpus luteum to produce progesterone to maintain an early pregnancy [23] and may also play other undiscovered roles. A recent meta-analysis found that low hCG detects SGA with a sensitivity of 34% at 90% specificity [16] suggesting it is unlikely to be useful as a lone marker for this disease, whilst other large studies that have not found any significant association [18,24].

Perhaps the most promising placenta-enriched protein for diagnostic purposes in recent times is placental growth factor (PlGF), a protein released from the syncytiotrophoblast. PlGF is a pro-angiogenic factor which is important in the pathogenesis of preeclampsia [25]. It has been shown to circulate at significantly lower concentrations in patients with established preeclampsia, and prior to the onset of the disease. In a seminal paper from Levine and others, women who eventually developed preeclampsia had significantly lower levels of PlGF relative to controls from 13 to 16 weeks onward [26]. Low second trimester circulating PlGF is associated with an odds ratio of 4.2 for developing severe, early onset preeclampsia – highlighting potential as a predictive biomarker [27,28]. Furthermore, PlGF may be useful in predicting babies who will be born SGA [29]. This suggests PlGF to be a marker of overall placental dysfunction rather than a marker of one particular disease.

While there is also an endothelial source, soluble Fms-like tyrosine kinase-1 (sFlt-1) is another placenta-enriched protein, and circulating levels rise sharply as pregnancy progresses. It is known to be significantly elevated in preeclampsia. sFlt-1 is now being used clinically in combination with PlGF as a 'rule-out' test for those women with a potential diagnosis of preeclampsia [30]. sFlt-1 is comprised of a number of splice variants [31,32], with the sFlt-1 e15a variant being reported as placenta-specific. Although commercial ELISAs measure a combination of sFlt-1 variants, we recently developed an e15a specific ELISA that may also have predictive potential [33]. Recent evidence also suggests that circulating levels of sFlt1/PlGF in combination with ultrasound measures may be a more accurate predictor of severe fetal growth restriction [34].

Thus, like mRNAs and miRNAs, placenta-enriched proteins hold much promise as potential diagnostics for placental diseases. Importantly, circulating proteins are readily detectable using common pathology techniques, such as ELISA-based methodology. Therefore, measurement of novel placenta-enriched proteins may be a gold-mine for discovering diagnostic markers of pregnancy diseases.

4. Targeting placenta-enriched molecules for therapeutic purposes

Diseases of pregnancy such as preeclampsia and fetal growth restriction currently do not have efficacious therapeutic options, however many potential therapies are now being considered [35–37]. Unfortunately, many small molecule drugs will cross the placenta to the fetus. This makes formulating new therapeutics a challenge due to the unknown and potentially harmful effects novel drugs may have on the fetus. The use of specific targeting towards placenta-enriched molecules may be a useful method of drug delivery in the future. This method could enhance delivery, minimise the doses of drugs required, and reduce fetal exposure to therapies. Study into targeting towards placenta-enriched molecules is already underway.

For example, our team have been focused on targeting highly expressed molecules on the placenta as a method to treat ectopic pregnancy. EGFR is a transmembrane glycoprotein and the founding member of the ErbB tyrosine kinase receptors [38]. What is perhaps not

widely known is that the placenta has the highest expression of EGFR compared to all other non-malignant human tissues [39]. It plays critical roles in placental development and survival. Thus, EGFR is in fact a placenta-enriched molecule. In light of this, the intentional use of EGFR inhibitors could effectively result in preferential targeting of the placenta – where it is most highly expressed. To this end, we have previously shown that the EGFR inhibitor gefitinib (when combined with methotrexate) potently regresses placental growth *in vitro* and *in vivo* [40]. This led us to undertake both phase I and phase II clinical trials. In using gefitinib to treat ectopic pregnancy, the phase II clinical trial combination therapy successfully resolved 86% (24/28) of ectopic pregnancies. This success has led to the initiation of a phase III randomised control trial being undertaken across the UK and Europe. This will compare the efficacy of the combined gefitinib-methotrexate approach versus methotrexate alone.

The use of nanoparticles for targeting drug therapy is popular through all fields of medicine. A successful nanomedicine must encapsulate a drug, avoid being destroyed in the circulation, and access the desired tissue where the drug can be released [41]. Doxorubicin packaged in non-targeted liposomes (Doxil) was the first FDA approved nanomedicine. It is a chemotherapeutic which passively targets malignant tumour cells [42]. Since its approval, nanomedicine has been a hot topic; more recently in reproductive biology.

For example, we have exploited the high placental expression of EGFR using nanoparticle therapy [43]. In preclinical studies, packaging doxorubicin into delivery vehicles decorated with EGFR-targeting antibodies enhanced placental demise *in vitro* and *in vivo* relative to non-targeted or naked drug. Interestingly, *Plasmodium falciparum* exploits a placenta-enriched mechanism in placental malaria. The parasite inserts a membrane protein (VAR2CSA) into the membrane of the erythrocyte which targets chondroitin sulfate chains present in the syncytiotrophoblast. Infected erythrocytes can therefore sequester in the placenta by adhering to these cells [44]. Utilising this same mechanism, methotrexate has been packaged into nanoparticles decorated with a synthetic placental chondroitin sulfate binding peptide [45]. The peptides have been shown to accumulate in the mouse placenta and *ex vivo* analysis confirmed placenta-specific delivery. Packaging of methotrexate specifically within these nanoparticles resulted in dramatic impairment of placental and fetal development, demonstrating effective delivery of a drug payload.

Tumour homing peptides specifically targeting integrins on the surface of the placenta [46] have also been used for placenta-specific targeting of nanoparticles. Lynda Harris and team have nicely demonstrated that placenta-peptide targeted nanoparticles accumulate in mouse placentae while control nanoparticles exhibit reduced binding and/or fetal transfer. When the nanoparticles were loaded with insulin-like growth factor 2, mice showed increased fetal and placental weights compared to controls suggesting effective delivery [46]. This team have also shown that they can effectively develop placental homing-peptide miRNA inhibitor conjugates [47]. In that work, miR inhibitors that have been identified as negative regulators of placental growth were conjugated to placental homing peptides. *In vivo* delivery resulted in significantly increased fetal and placental weights relative to controls [47]. All of these studies demonstrate the potential utility of targeting nanoparticles to placenta-enriched molecules in order to treat placental diseases.

Of course, a key consideration when utilising placenta-targeted nanomedicine is ensuring any particles crossing the placenta are not detrimental to the fetus. For review around the importance of surface charge and composition in determining whether nanoparticle cross the placenta or not see Muoth et al. [49]. A recent Japanese mouse model demonstrated that smaller nanoparticles (~70 nm) could pass through the placenta and were detectable in the fetal liver and brain. Larger particles (300 and 900 nm) did not reach the fetus [48]. The placentae of mice treated with smaller nanoparticles exhibited abnormal blood flow, with increased rates of fetal growth restriction and fetal death in utero. This study highlights the importance of formulating nanoparticles that induce no harm on the fetus in pregnancies that are ongoing.

As well as offering a potential biomarker for preeclampsia, the placenta-enriched molecule sFlt-1 has also been a target of therapeutic studies. It is hypothesised that reducing circulating levels of this molecule may be of therapeutic benefit in prolonging preeclamptic pregnancies. In many studies from our own laboratory, we have demonstrated that small-molecule inhibitors such as metformin, esomeprazole and statins, can reduce placental secretion of sFlt-1 [35–37]. In other work, sFlt-1 has been removed via extra-corporeal or dextran sulfate apheresis [50,51]. Recently, direct targeting of sFlt-1 has been demonstrated via siRNA therapies administered to a baboon preeclampsia model [52]. A single dose of siRNAs targeting the three sFlt-1 mRNA isoforms thought to be responsible for placental sFlt-1 overexpression in disease was used. This effectively suppressed sFlt-1 overexpression, and clinical signs of preeclampsia [52]. This work demonstrates that placenta-enriched molecules may also be directly targeted via RNA interference. A better understanding of the many placenta-enriched molecules, their biology and contribution to disease pathogenesis is highly likely to reveal further potential therapeutic targets.

5. Conclusion

There is growing evidence that placental health has short (i.e. pregnancy specific) and long-term effects on the developing fetus. Many of these are yet to be uncovered. The placental transcriptome and proteome are foreign to the mother. Exploiting the differences, by detecting placenta-specific gene products in the maternal circulation, is an important minefield for discovery of non-invasive biomarkers of placental disease. Excitingly, recent research utilising placenta-specific targeting as a therapeutic option suggests this approach has potential for novel treatments of pregnancy disorders. Clearly, we can identify changes in placental factors within the maternal circulation; potentially we can use this information to better understand diseases of pregnancy, and develop targeted approaches to correct them.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.02.002.

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