# A tissue-based proteomic study of VEGFR2 in human term placentas revealed its association with pyruvate dehydrogenase and MDMX

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**ABSTRACT** VEGFR2 is the main regulator of placental angiogenesis and at term is localized in endothelial cells (EC) of the villous vasculature. VEGFR2 immunoprecipitation (IP) of membrane proteins, extracted from the fetal compartment, isolated 30 proteins that were identified by proteomic analysis. Among them are pyruvate dehydrogenase (PD) component-E2 (DLAT), heterogeneous nuclear ribonucleoproteins, annexins, MDMX and titin. Some of these interactions would involve the "nuclear" VEGFR2 since VEGFR2 and PD translocate to the nucleus. Ingenuity pathway analysis of VEGFR2-IP predicted association of 11 proteins, not MDMX or titin, with tretinoin-mediated signaling (overlap p-value $8X10^{-7}$) potentially expanding with the retinoid family the list of proteins and mechanism of the VEGFR2 signaling pathway.

## BACKGROUND

### Pyruvate Dehydrogenase (PD).

Under normal aerobic conditions, pyruvate is transported into mitochondria and converted to acetyl-CoA by the PD complex consisting of pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2/**DLAT**), dihydrolipoamide dehydrogenase (E3), E3-binding protein (E3BP/**PDHX**), and two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP).

### MDMX, MDM2 and p53.

**p53**, the "guardian" of the human genome which is mutated in all cancers, regulates a variety of cellular processes, including DNA repair, apoptosis, cell cycle arrest, senescence, autophagy and metabolism. In preeclampsia, p53 is upregulated in the trophoblast region, due in part to hypoxia and oxidative stress.[1]

MDM2 and MDM4 are the main regulators of p53. They bind to and inhibit the transactivation domain of p53, cooperate to poly-ubiquitinate p53 and facilitate its translocation to the cytosol. MDM2 and MDMX are essential for regulating p53 levels during development.[2] In breast cancer, MDM2 and MDMX bind to and downregulate ERα independently of p53.[3] Targeting MDMX in pharmacological reactivation of wild type p53 in tumors is more effective and less toxic to the patients than MDM2.

**OBJECTIVE**: To isolate by immunoprecipitation, identify and study proteins in the fetal compartment of placentas that associate with, a) The extracellular domain of VEGFR2 and may impact reactions regulated by VEGFR2, and b) DLAT.

**METHODS**: We collected placental samples from 32 patients at term with a wide range of clinical presentations, parity, labor, BMI, preeclampsia, diabetes, but no IUGR complications. Tissues stored at -80$^0$C were processed as outlined on **Fig 1**. In VEGFR2 immuno-precipitations (IP) the magnetic beads were charged with antibody-2479 from Cell Signaling. Proteins bound to the extracellular domain of placental VEGFR2 would be selected since antibody-2479 was raised to a C-terminus peptide of human VEGFR2. In DLAT-IP the SC-32925 DLAT antibody was used. Mass spectrometry identified the immunoprecipitated proteins.

**RESULTS**: Among the proteins identified in VEGFR2 IP were the pyruvate dehydrogenase E2-component (PDC-E2 or DLAT), MDMX and many others, as shown on **Table 1**. DLAT-IP did not pull down VEGFR2, but isolated numerous other proteins and many members of the PD system (**Table 1**). Where do these IP associations occur? Some proteins that coIP with VEGFR2 and DLAT are likely to reside in the EC nucleus where VEGFR2 and DLAT can translocate (**Fig 3**). Only MDMX (not MDM2 or p53) was found in VEGFR2-IP and DLAT-IP experiments.

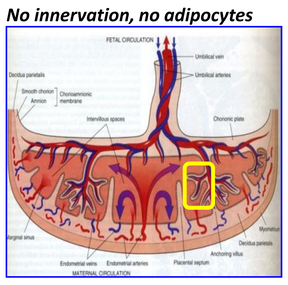
Ingenuity pathway analysis of VEGFR2-IP predicts an association of 11 proteins (**Table 1**) with tretinoin-mediated signaling (p-value of overlap $8X10^{7}$).

VEGFR2 catalyzes the formation of dinucleoside polyphosphates (e.g. $Up_4A$) which, like ATP, are ligands of purinergic receptor P2Y2. Western blots of the VEGFR2-IP eluates revealed coIP of P2Y2, not identified by proteomic analysis of the eluates, after trypsin digestion, probably because P2Y2 is very basic (pI=9.6) and may be digested extensively.

On the **Scheme**, solid arrows point to some of the proteins on **Table 1** that coIP with VEGFR2. Mitochondrial Sirtuin-4 binds to DLAT and possesses lipoamidase activity[4]. If lipoamidase Sirtuin-4 is activated in the placenta it could have serious consequences for mitochondrial acetyl-CoA formation and ATP synthesis. Only a few proteins are lipoylated, but they function in cellular metabolism are targets of SIRT4.

The central region of placental MDMX must be post-translationally modified impeding the binding of antibodies made to that region (**Fig 4**). Bethel antibody to the N-terminus recognized a 78 kDa band in western bonds; both p53 and MDM2 bands are seen at 50-54 kDa.

**Fig. 1**




Samples of the **fetal** compartment are stored at -80$^0$C

**Tissue homogenates are spun at 100,000g**

**Pellets** contain, villous plasma membranes, mitochondria, nuclei, exosomes, endothelial progenitor cells and other blood cells from the mother and fetus

Pellets are extracted with ASB-14. Extracts are spun at 100,000g

**The 100,000g supernatants contain solubilized membrane proteins and are used in immunoprecipitations**

**Supernatants** contain soluble proteins, mother & baby's blood, antibodies (Ab) and **auto**-Ab

*% of proteins solubilized by ASB-14: VEGFR2 97; MDMX 100; PDC-E2 86 and TOM20 64.*

**Table 1**

| Proteins that co-immunoprecipitate (coIP) with VEGFR2 | | | CoIP with DLAT |
|---|---|---|---|
| | Autoimmune disease antigens (blue) and/or associated with tretinoin signaling (red) | | |
| ID | Symbol | Entrez Gene Name | |
| P04083 | ANXA1 | Annexin A1 | √ |
| B4DVE7 | ANXA11 | Annexin A11 | √ |
| P07355-2 | ANXA2 | Annexin A2 | √ |
| B4DHY4 | ANXA7 | Annexin A7 | √ |
| P02745 | C1QA | Complement component 1, q subcomponent, A chain | |
| P10515 | DLAT | Dihydrolipoamide S-acetyltransferase (*Primary Biliary Cholangitis*) | √ |
| Q9UBS4 | DNAJB11 | DnaJ (Hsp40) homolog, subfamily B, member 11 | |
| Q9Y6R7 | FCGBP | Fc fragment of IgG binding protein | |
| P09651 | HNRNPA1 | Heterogeneous nuclear ribonucleoprotein A1 | |
| P22626-2 | HNRNPA2B1 | Heterogeneous nuclear ribonucleoprotein A2/B2 (*Vasculitis*) | |
| P51991 | HNRNPA3 | Heterogeneous nuclear ribonucleoprotein A3 | √ |
| D6RAF8 | HNRNPD | Heterogeneous nuclear ribonucleoprotein D | √ |
| B4DHY1 | HNRNPH3 | Heterogeneous nuclear ribonucleoprotein H3 (2H9) | |
| P01876 | IGHA1 | Immunoglobulin heavy constant alpha 1 | |
| P01857 | IGHG1 | Immunoglobulin heavy constant gamma 1 (G1m marker) | |
| P01860 | IGHG3 | Immunoglobulin heavy constant gamma 3 (G3m marker) | |
| P01871-2 | IGHM | Immunoglobulin heavy constant mu | |
| P01834 | IGKC | Immunoglobulin kappa constant | |
| B9A064 | IGLL1/IGLL5 | Immunoglobulin lambda-like polypeptide 1 | |
| P35968 | VEGFR-2/KDR | Kinase insert domain receptor | |
| P41231 | P2Y2 | Purinoceptor 2 | |
| Q99572 | P2X7 | Purinoceptor 7 | |
| A6NIW5 | PRDX2 | peroxiredoxin 2 (*Vasculitis*) | |
| Q6NZI2 | PTRF | Cavin-1 | |
| P62701 | RPS4X | Ribosomal protein S4, X-linked | |
| O95810 | SDPR | serum deprivation response | |
| Q2F832 | SSB | Sjorgen syndrome antigen B (*autoantigen La*) | |
| Q8WZ42 | TTN | Titin | √ |
| P10155 | TROVE2 | TROVE domain family, member 2 | |
| P08670 | VIM | Vimentin (*Citrulinated-VIM is AA for Rheumatoid arthitis*) | √ |
| P21980 | TGM2 | Tissue transglutaminase (*celiac disease*) | |
| O15151 | MDM4 | HDMX (human double minute 4) | √ |

Gene names of proteins that coIP with DLAT, but are not checked on Table 1: C1QA, C1QC, IGHM, α2M, Actin, LGALS3, KIF20B, PDHA1, PDHB, PDHX, DLD, TRIM21.

**Table 2**

Patients #3 and #4 are preeclamptic

| Pts | Age (y) | BMI | DM | Sex F=1 | Baby wt | c/s=2 | Gravidy |
|---|---|---|---|---|---|---|---|
| H1 | 25 | 30.0 | 0 | 1 | 2940 | 1 | 1 |
| H2 | 21 | 24.0 | 0 | 2 | 2590 | 1 | 2 |
| H3 | 31 | 32.0 | 0 | 1 | 3040 | 1 | 2 |
| H4 | 24 | 30.0 | 0 | 1 | 1965 | 2 | 2 |
| I1 | 24 | 34.0 | 0 | 2 | 2631 | 2 | 3 |
| I2 | 21 | 30.0 | 0 | 2 | 3759 | 1 | 1 |
| I3 | 30 | 34.0 | 1 | 2 | 3195 | 2 | 8 |
| J1 | 27 | 25.0 | 0 | 2 | 3300 | 2 | 2 |
| J2 | 38 | 22.0 | 0 | 1 | 3460 | 1 | 2 |
| J3 | 28 | 28.0 | 0 | 2 | 3630 | 2 | 3 |
| J4 | 19 | 41.0 | 0 | 1 | 3335 | 1 | 1 |
| L1 | 25 | 24.0 | 0 | 1 | 3147 | 2 | 1 |
| L2 | 19 | 29.0 | 0 | 1 | 2920 | 1 | 1 |
| L3 | 24 | 30.0 | 0 | 2 | 2235 | 2 | 3 |
| L4 | 30 | 29.0 | 0 | 1 | 3010 | 1 | 3 |
| M1 | 25 | 55.0 | 0 | 1 | 3795 | 2 | 3 |
| M3 | 20 | 31.3 | 0 | 1 | 3490 | 2 | 2 |
| M4 | 26 | 39.8 | 0 | 2 | 3380 | 1 | 1 |
| N1 | 32 | 30.0 | 1 | 2 | 3800 | 1 | 4 |
| N2 | 33 | 32.8 | 0 | 1 | 3180 | 2 | 2 |
| N3 | 33 | 35.5 | 0 | 2 | 3390 | 2 | 2 |
| N4 | 19 | 29.5 | 0 | 2 | 3510 | 2 | 2 |
| O1 | 23 | 26.2 | 0 | 1 | 2960 | 1 | 2 |
| O2 | 24 | 34.8 | 0 | 1 | 2940 | 1 | 1 |
| O3 | 28 | 29.5 | 0 | 1 | 2800 | 1 | 1 |
| O4 | 25 | 52.5 | 0 | 2 | 3575 | 2 | 3 |
| O5C | 36 | 50.4 | 1 | 2 | 4295 | 2 | 4 |
| O6 | 34 | 62.6 | 1 | 2 | 4440 | 2 | 3 |
| O7 | 32 | 35.2 | 0 | 1 | 3780 | 2 | 12 |
| O8C | 28 | 27.3 | 0 | 1 | 3605 | 2 | 3 |

**Fig. 2**

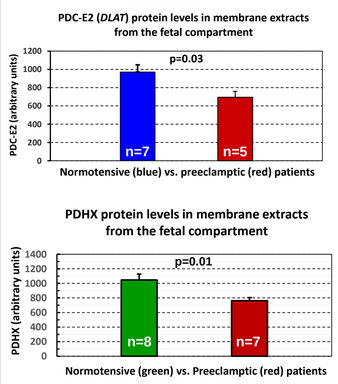
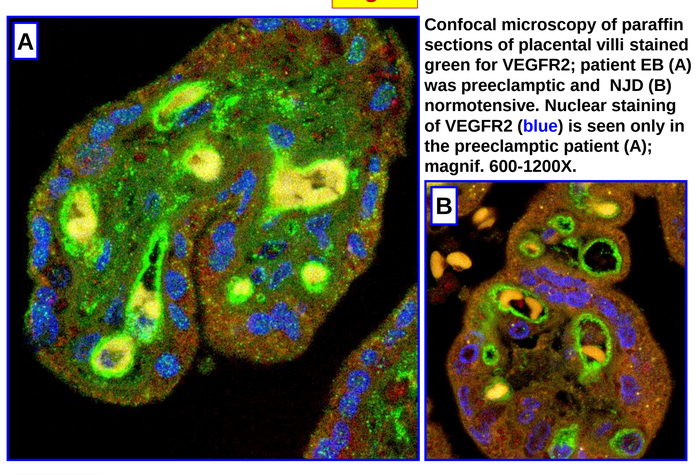
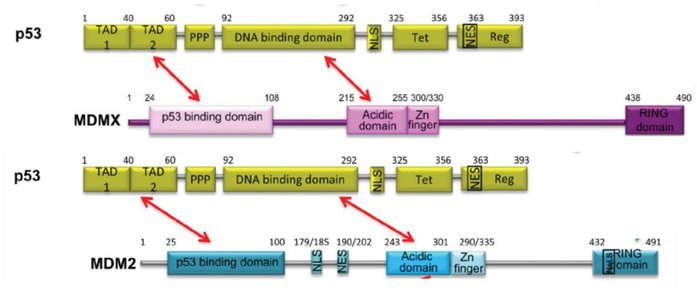



**Fig. 3**




**Confocal microscopy of paraffin sections of placental villi stained green for VEGFR2; patient EB (A) was preeclamptic and NJD (B) normotensive. Nuclear staining of VEGFR2 (blue) is seen only in the preeclamptic patient (A); magnif. 600-1200X.**

**Fig. 4**




Epitopes of commercial antibodies for western blots of placental extracts.
**MDM4** 125-175 (Bethyl); **MDM2** 154-167 (Santa Cruz); **p53** 11-25 (Santa Cruz)

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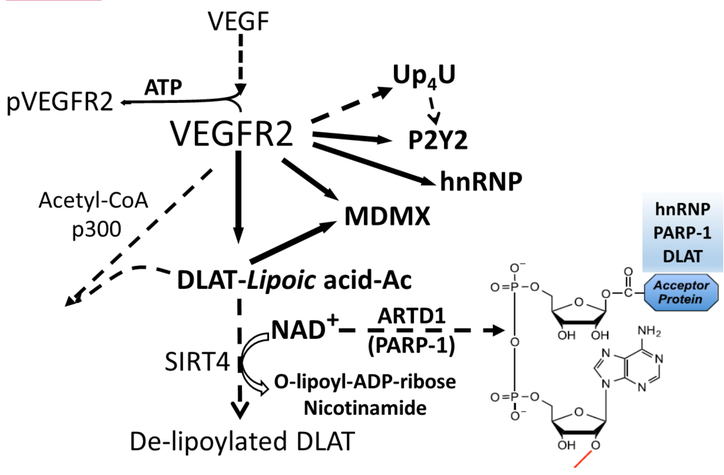
3. **Swetzig WM et al. Estrogen receptor alpha (ERalpha/ESR1) mediates the p53-independent overexpression of MDM4/MDMX &MDM2 in human breast cancer. Oncotarget 2016;7:16049-69.**

**Scheme**




## DISCUSSION.

The biochemical approach we used to discover new regulators of placental angiogenesis was centered on identifying proteins linked to VEGFR2, the main regulator of placental vasculogenesis and angiogenesis. By refraining from extensive tissue fractionation, we sought to retain proteins weakly associated with VEGFR2.

A wealth of information was gathered from our study of VEGFR2 in "crude" preparations of the fetal compartment of term placentas. Assuming that co-immunoprecipitation (coIP) of proteins with VEGFR2 or with DLAT translates to a previously unknown functional interaction, we may have found new links between angiogenesis and enzymes metabolizing pyruvate in normotensive and preeclamptic patients. The recently found co-immunoprecipitation of MDMX with VEGFR2 and with DLAT offers intriguing opportunities to explore in the context of genome stability.

We assume that proteins that coIP with VEGFR2 (Table 1) are localized on EC. However, plasma cell markers (IGKC, IGHM, IGAH1)[5] were found to coIP with VEGFR2. This implies that proteins from VEGFR2$^+$ cells in mother and baby's blood, that "contaminates" our preparations (**Fig 1**), are also captured. Does this finding imply a crosstalk between metabolism and immunity in the placental? [6]

The reported association between DLAT and SIRT4 and the lipoamidase activity of SIRT4[4] poses a dangerous scenario of limiting ATP availability during gestation and labor. The preliminary finding (**Fig 2**) that DLAT and PDHX protein levels are decreased in preeclampsia will be studied to clarify the level of their post-translational modifications (delipoylation, acetylation, ADP-ribosylation) that may contribute to this observation.

Since severe intrahepatic cholestasis of pregnancy (ICP) is a risk factor for preeclampsia, it is not unreasonable to propose a role for the liver in preeclampsia through the pyruvate dehydrogenase E2-component, DLAT, which is established as the "Achilles heel" of primary biliary cholangitis (PBC). In severe PBC, autoantibodies to DLAT cause the destruction of the biliary tree that requires liver transplantation. ICP is not as severe for the mother as for the fetus which shows increased PT heart tracing with severe sequelae in utero and the NICU. The association of placental titin with VEGFR2 will be explored considering that titin mutations are associated with heart disease.

## CONCLUSIONS:

- ❑ Some proteins that co-immunoprecipitated with VEGFR2, highlighted in blue on Table 1, are antigens for autoimmune diseases although none of our patients presented with such symptoms. Do these associations with VEGFR2 imply a vascular component of these diseases and signal predisposition of the child or the parents to develop them in the future?
- ❑ VEGFR2 intracellular trafficking (translocation) would account for its associations in vivo with proteins not confined to the plasma membrane of endothelial cells in the villous vasculature.
- ❑ It is likely that endothelial progenitor cells, immune cells and other components of mother and baby's blood could participate in the signal transduction system of VEGFR2 during pregnancy.
- ❑ We hypothesize that in the large list of suspected contributors to preeclampsia will be included members of the pyruvate dehydrogenase complex along with proteins interacting with it, namely, Sirtuin-4 and PARP-1.

**REFERENCES** (con'd)

4. **Mathias RA et al. Sirtuin 4 is a lipoamidase regulating pyruvate dehydrogenase complex activity. Cell 2014;159:1615-25.**
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6. **Man K et al. Tissue Immunometabolism: Development, Physiology, and Pathobiology. Cell Metab 2017;25:11-26.**