Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 01/01/2009 – 12/31/2010

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl A. Richards, MBA

4. **Grant Contact Person's Telephone Number:** 412-641-8932

5. **Grant ME Number or SAP Number:** 4100047639

6. **Project Number and Title of Research Project:** Project 7 - Analysis of Functional Domains within NDRG1

7. **Start and End Date of Research Project:** 01/01/2009-12/31/2010

8. **Name of Principal Investigator for the Research Project:** Yoel Sadovsky, MD

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 244,013.04

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Sadovsky	PI	10%	$23,846.09
Shi	Research Instructor	100%	$66,064.39
Phillips	Research Lab Manager	100%	$15,118.88
Jennings	Research Assistant	100%	$15,274.41

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Rideout	Research Administrator	1

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Placental Injury and Adaptation	☒ NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	July 2009	$1,949,021	$ Pending re-submission
The Role of NDRG1 in Placental Injury (Training grant for Dr. Jacob Larkin, with Dr. Sadovsky as mentor)	☐NIH ☐ Other federal (specify:_____ _____) ☒ Nonfederal source (specify: American Association of Obstetricians & Gynecologists Foundation)	July 2009	$300,000	$300,000

	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

As noted above, we have submitted an NIH R01 proposal, which received a favorable score and are resubmitting in March 2010.

12. Future of Research Project. What are the future plans for this research project?

Once funding is obtained we will continue our pursuit to determine the mechanism of action of NDRG1, and expand our analysis to NDRG- interacting proteins. We will also include an in vivo analysis of NDRG1 function.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1			1
Female				
Unknown				
Total	**1**			**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	1			1
Unknown				
Total	**1**			**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White	1			1
Black				
Asian				
Other				
Unknown				
Total	**1**			**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No____X_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes_____ No____X_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. **Collaboration, business and community involvement.**

    16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

    Yes_____X_____ No_____

    If yes, please describe the collaborations:

    We improved our ties with Center for Biological Imaging at the University of Pittsburgh, which allowed us to perform advanced imaging and quantification of placental proteins.

    16(B) Did the research project result in commercial development of any research products?

    Yes_____ No_____X_____

    If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the entire grant award period. Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Research Objectives

This proposal centers on a protein called N-myc downstream regulated gene 1 (NDRG1), which plays an important role in regulation of cell proliferation, differentiation and apoptosis in diverse cell types. In several types of cancer cells, NDRG1 reduces the rate of cell

proliferation and promotes programmed cell death. NDRG1 is also important in the function of Schwann cells. In our laboratory we found that NDRG1 expression is markedly increased in placental trophoblast cells that are exposed to hypoxia. Importantly, using lentivirus-mediated over-expression of NDRG1 we showed that NDRG1 attenuates hypoxic injury in primary human trophoblasts. As expected, silencing of NDRG1 in primary human trophoblasts led to enhanced hypoxia-induced apoptosis.

Although pertinent to several cellular processes, the mechanism of action of NDRG1 remains unknown. Computerized analysis suggested that NDRG1 belongs to a family of protein hydrolases, yet it lacks a hydrolase activity. This application serves as a first attempt to dissect the function of putative domains within NDRG1. In this grant proposal we hypothesize that discrete domains within NDRG1 determine the function of this protein in cellular differentiation and apoptosis. We will (a) use deletion mutagenesis to create NDRG1 proteins that are deficient in discrete domains, and assess their influence on cell proliferation in a cell line, and (b) dissect the specific functions of identified NDRG1 domains in the context of primary human trophoblasts.

Our research targets the function of NDRG1 primarily in human trophoblast. Understanding the function of NDRG1 in the placenta will illuminate pathways that underline placental adaptation to injury. Placental dysfunction is the most important cause of fetal growth restriction, a disease associated with increased perinatal morbidity and mortality. Moreover, surviving children are susceptible to cerebral palsy and growth delay, and as adults they are at greater risk for acquiring metabolic syndrome. Lastly, while our research targets the placenta, results from our proposed experiments have a direct relevance to cancer and attenuation of tumor growth.

Specific Aims

Fetal growth restriction (FGR) results from failure of the developing fetus to reach its pre-determined growth potential. FGR is associated with increased perinatal morbidity and mortality. Surviving children are susceptible to cerebral palsy, growth delays and neurobehavioral abnormalities. Growth restricted individuals that survive childhood are at increased risk for the metabolic syndrome in adulthood. A common pathway of FGR is reduced placental perfusion, which leads to decreased nutrient supply, cellular hypoxia and an increased rate of apoptosis. We recently found that N-myc downstream regulated gene 1 (NDRG1), a widely expressed gene that regulates cell proliferation, cellular differentiation, and apoptosis, is up-regulated in placentas from pregnancies complicated by FGR, as well as in human trophoblasts cultured in hypoxic conditions. Moreover, we used two complementary approaches to show that NDRG1 protects primary term human trophoblasts from hypoxic injury: (a) NDRG1 over-expression diminishes hypoxia-induced apoptosis and promotes cell differentiation in cultured primary human trophoblasts, and (b) silencing of NDRG1 in primary human trophoblasts leads to enhanced hypoxia-induced apoptosis. Whereas these data provide mechanistic and relevant support to the role of NDRG1 in human placental cells, we do not understand how the protein NDRG1 works. *We hypothesize that discrete domains within NDRG1 determine the function of this protein in cellular*

proliferation, differentiation, and apoptosis. The two aims described below will enable us to test our hypothesis:

Aim I: Create mutations within NDRG1 protein and assess their influence on proliferation in a cell line.
Aim II: Test for specific functions of the identified NDRG1 domains in primary human trophoblasts.

Progress in Achieving Research Goals, Objectives and Aims

Aim I: Create mutations within NDRG1 protein and assess their influence on proliferation in a cell line.

We initially used several research tools to conduct an extensive *in silico* analysis of putative functional domains within NDRG1. We also examined the conservation of these domains in other NDRG family members. We noted that the similarity of NDRG1 with other family members is markedly reduced at the N- and C-termini of the protein. Our analysis identified the following domains within NDRG1: (a) a sequence between aa 21-42 of the N-terminus, identified as a putative DNA-binding helix-turn-helix (HTH) domain, and is not conserved in the other NDRG family members, (b) an alpha/beta hydrolase (acyltransferase) superfamily domain at residues 34-316, and an alpha/beta hydrolase fold at residues 89-167, both identified using a BLAST homology search of NDRG1 against whole proteins or discrete domains, (c) a phosphopantetheine attachment site, at residues 128-143, identified using EMBL-EBI databases, (d) a C-terminal repeat (aa 339-368) of three GTRSRSHTSE sequences, which is not conserved in the other members of the NDRG family. Using refseq-protein and swissprot scans (NCBI) we found homologous domains in several proteins.

We emphasize that at the present time, except for the observation that NDRG1 does not exhibit hydrolase activity, there is no data on the mechanism of action of NDRG1 or on the function of specific NDRG1 domains. In order to investigate the functional role of these and other NDRG1 domains we generated the following NDRG1 deletion mutants, all cloned into a pcDNA 3.1a vector upstream from a C-terminal myc tag, designed to enable detection of the mutant NDRG1 protein and distinguish it from the endogenous, untagged protein. These truncated proteins include:

a. NDRG1-del2-42, which is deleted for a large portion of the N-terminus (including the putative DNA-binding HTH domain), and designed to assess if unique N-terminal sequences, which are not conserved in other NDRG family members, are essential.
b. NDRG1-del21-42, which is deleted for the putative DNA-binding HTH domain at the protein's N-terminus.
c. NDRG1-1-317, which is deleted for the NDRG1 unique C-terminus (including several phosphorylation sites), and designed to assess if unique C-terminal sequences that are not conserved in other NDRG family members, are functionally important.

d. NDRG1-del339-368, which is deleted for the three C-terminal repeats (three repeats of the RSRSHTSEGT motif). As we noted earlier, this motif is conserved in a number of proteins that are involved in diverse metabolic functions including cell cycle regulation and apoptosis. These domains in the C-terminal region of NDRG1 were also shown to effectively bind nickel.
e. NDRG1-del128-143, which deletes the phosphopantetheine attachment site (PPAS). This domain serves as a potential nuclear localization signal, and is conserved in several acyl carrier proteins.

Each construct described above was produced using a one- or two-way ligation of PCR products, and the cDNA fragment was cloned between NheI and HindIII of the plasmid vector, with an in-frame myc-epitope tag downstream of the multiple cloning site. Each construct was sequenced, effectively expressed in the trophoblast lines JEG3, BeWo and HTR8, and detected using western blotting with an anti-myc antibody (see Fig. 1 for representative western blot).

Trophoblast derived cell lines are useful for analysis of protein function, as they are relatively easy to manipulate and to assess for functions such as cell proliferation. Certain cell lines even retain certain differentiated functions of primary trophoblasts (e.g., production of hCG, syncytium formation). Because we initially planned to test the function of NDRG1 in trophoblast lines, we sought to ensure that NDRG1 was indeed expressed in the relevant cells (BeWo, JEG3 and HTR8). Using primary human trophoblasts as a positive control and the CHO ovarian cell line as a negative control, we found that NDRG1 was indeed expressed in these cells (Fig. 2). We also ensured that we can over-express NDRG1 proteins in our relevant cell lines (Fig. 3).

In our initial functional assays we examined the effect of over-expression or silencing of NDRG1 on cell proliferation. We over-expressed NDRG1 using polyethylenimine (PEI), and tested for cell proliferation at 48, 72, or 96 hours using the MTS assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell proliferation Assay, Promega). For silencing, we used an siRNA (100 pmol of siRNA oligo per well in a 6-well plate) that specifically targeted human NDRG1 and compared its effect to that of non-specific control siRNA (both from Invitrogen). The siRNA oligonucleotides were introduced using Lipofectamine 2000 (Invitrogen). Fig. 4 demonstrates our ability to overexpress or silence NDRG1. Cell proliferation was measured by MTS assay after transfection. As shown in Fig. 5, over-expression or silencing of NDRG1 had no effect on cell proliferation.

In order to carry out our proposed analysis of NDRG1 domains, we elected to use the cell lines for assessment of two cellular functions that are relevant to primary human trophoblasts, namely cell differentiation and apoptosis. We first demonstrated that pertinent assays designed to uncover the function of mutant NDRG1 proteins are functional in our cell lines in a manner similar to our previous findings in primary human trophoblasts. As shown in Fig. 6A-B, addition of the hypoxia mimetic cobalt chloride (CoCl, 25-200 mM) or exposure to UV light (0.1-10mj/cm^2) enhanced apoptosis, detected by caspase 3 activity, akin to the effect of hypoxia (FiO_2=1%) on apoptosis, as we previously demonstrated. Importantly, the effect of hypoxia on apoptosis was effectively potentiated by siRNA-mediated silencing of NDRG1 (Fig. 6C). We also demonstrated that BeWo cells underwent differentiation, determined by syncytia formation and hCG production, in the presence of the protein kinase A activators forskolin or 8-Br-cAMP

(Fig. 7). Whereas over-expression of NDRG1 had no effect on this differentiation process, silencing of NDRG1 attenuated the differentiation of BeWo cells, as shown by the release of hCG, a marker of cell differentiation, to the culture medium (Fig. 8). The impact of UV light on apoptosis was also potentiated by silencing of NDRG1 (Fig. 9).

In order to enable us to over-express a mutant NDRG1 protein and test its effect on rescue of wild type NDRG1 function, we developed a double transfection strategy, where we analyzed the effect of simultaneous silencing of NDRG1 and over-expression of wild type or mutant myc-tagged NDRG1. Using this combined approach, we found that UV light induced apoptosis in cells transfected with either control or NDRG1 siRNA. Over-expression of wild type NDRG1 attenuated the effect of UV light on JEG3 apoptosis. Importantly, among the different NDRG1 constructs, NDRG1 that was deficient of the N-terminal HTH domain (del 2-42 mutant) was unable to attenuate the effect of UV light on JEG3 apoptosis (Fig. 10). Similarly, whereas over-expression of wt NDRG1-myc rescued cell survival in hypoxic cells in which NDRG1 was silenced, this "protective effect" of NDRG1 was abolished in cells that over-expressed NDRG1-del2-42.

Taken together, our data suggest that NDRG1 protects trophoblast cell lines against injury induced by hypoxia, CoCl and UV light, and enhances cell differentiation. The effect of NDRG1 is particularly noticeable during cell injury, with little effect at the basal state. The high level of endogenous NDRG1 requires the use of siRNA-mediated silencing in order to uncover the effect of NDRG1. Over-expressing NDRG1 after knock down of this protein can rescue cell phenotype, and this model may be suitable to assess the function of NDRG1 that harbors a deletion mutation in a putative functional domain. Our data thus far suggest that the amino terminus domain of NDRG1 (aa 2-42) harbors key functional domains, which are obligatory for intact function of NDRG1. Current experiments target the ability of NDRG1 mutants to restore NDRG1 function in injured cells, as well as during BeWo cell differentiation.

Aim II: Test for specific functions of the identified NDRG1 domains in primary human trophoblasts.

We found that exposure of JEG3 cells to either hypoxia or CoCl caused a change in cellular localization of either native NDRG1 or over-expressed NDRG1-myc recombinant protein, with enhanced localization in the nucleus and cell membrane, and less in the cytoplasm (Fig. 11). In light of these findings, we sought to assess whether or not this response was reproducible in primary human trophoblasts, which form multinucleated cells (syncytiotrophoblasts) as they differentiate in vivo and in vitro. We exposed primary human trophoblasts to hypoxia in our Forma Scientific hypoxia chamber, designed to maintain a constant level of pre-determined FiO2. As shown in Fig. 12, exposure of primary human trophoblasts to hypoxia for 24-72 h resulted in altered intracellular expression of NDRG1, with enhanced localization of NDRG1 to the nucleus and a concomitant decrease in cytoplasmic expression of NDRG1. These changes were not observed in cells exposed to standard conditions (not shown). We quantified the nuclear localization of NDRG1 using two approaches. First, we counted the number of nuclei that expressed NDRG1. For that purpose we first transfected cells with an NDRG1 expression vector, and counted NDRG1-positive cells when exposed to the hypoxia-mimetic CoCl vs control. As shown in Fig. 13A, exposure of cells to the hypoxia-mimetic CoCl caused more localization of

NDRG1 in the nucleus, compared to controls cells. This was quantified using nuclei count (Fig. 13B), revealing a greater fraction of NDRG1-positive nuclei in exposed trophoblasts. In our second approach, we used cell fractionation and extracted nuclei and cytoplasm fractions from cells cultured in normal condition, hypoxia, or from cells treated with CoCl. We examined NDRG1 expression in these fractions using immunoblotting. As expected, we found that more NDRG1 was expressed in the nuclear fraction of cells exposed to CoCl or incubated in hypoxia (FiO_2 <1%) for 24 hours (Fig. 14).

Results from experiments described in Aim 1 necessitated that we find an approach that would allow us to simultaneously silence endogenous NDRG1 (using siRNA) while over-expressing exogenous wild type or mutant NDRG1 in primary human trophoblasts. This became essential even prior to the use of lentiviruses. After testing several preparations, we elected to use DharmaFECT-2 (Dharmacon), which is designed for co-expression of short silencing oligonucleotide along with a protein-encoding expression vector. Using transfections in 6-well plates with antibiotic-free medium, we found that DharmaFECT-2 was effective for co-expressing siRNA and an expression vector, without any evidence for cell toxicity. This therefore has become our working tool while we deploy a lentivirus system in primary trophoblasts.

While we made significant progress in our experiments, we did not fully complete the desired lentivirus-related goals, primarily in Aim II. This largely stemmed from the fact that we had to develop additional means to assess NDRG1's function in cell lines, after disappointing results using cell proliferations assays, and the need for substantial effort to identify the function of NDRG1 in the context of cell differentiation and apoptosis. Moreover, we developed a new experimental strategy that was based on combined RNA interference and concomitant plasmid transfection to rescue the function of NDRG1.

Overall, we believe that the experiments advanced our knowledge of the function of NDRG1 during trophoblast adaptation to injury. Our results validated the necessary technologies and served to define cellular functions that are directly influenced by NDRG1. We have created a series of mutated NDRG1 proteins and have found a deletion mutant that cannot rescue the action of NDRG1. We are now poised to perform additional experiments that are far more sophisticated than those originally intended and proposed, and focus our work on primary cells. We believe that understating the molecular actions of NDRG1 will shed light on the means to regulate the activity of this important protein during trophoblast injury that may take place in the course of human pregnancy.

Figures and figure legends

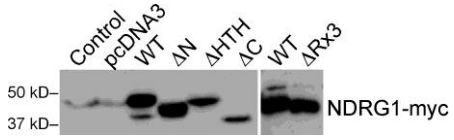

Fig. 1: Western immunoblotting showing the expression of NDRG1 deletion mutants, fused to myc-tag at the C-terminus. Detection was performed with an anti-myc antibody.

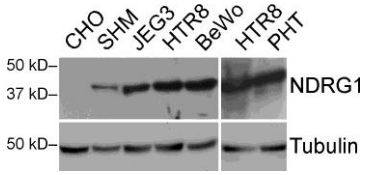

Fig. 2: Western immunoblotting showing the expression of wt NDRG1 in relevant cell lines: Primary human trophoblasts (PHTs, serving as a positive control), BeWo, HTR8 and JEG3 are human trophoblast cell lines that are relevant to our experimental plan, Syrian hamster myocytes (SHM), Chinese hamster ovary cells (CHO), serving as a negative control. Detection was performed with an anti-NDRG1 antibody.

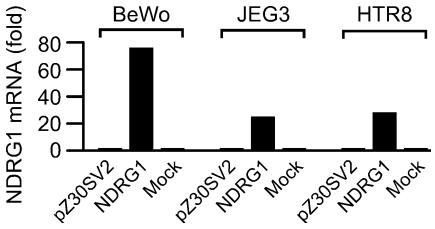

Fig. 3: The expression level of NDRG1in relevant cell lines, assessed using RT-qPCR, after over-expression of a wt NDRG1 plasmid,

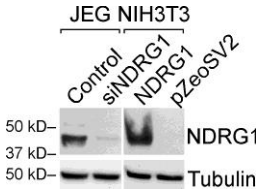

Fig. 4: Western immunoblotting of NDRG1, with efficient silencing of NDRG1 in JEG3 cells, which express NDRG1 endogenously (left lanes), and overexpression of NDRG1 in NIH3T3 cells, which lack endogenous NDRG1 (right lanes). Detection was performed with an anti-NDRG1 antibody

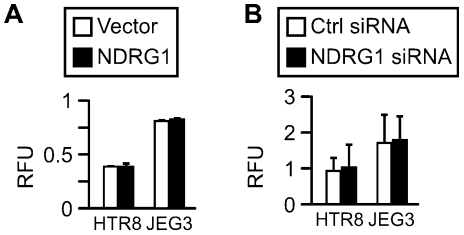

Fig. 5: The effect of NDRG1 over-expression (panel A) or silencing (panel B) on proliferation of HTR8 or JEG3 cells. Proliferation was determined using an MTS assay, with absorbance of formed aqueous soluble formazan product determined at 490 nm using the Versa Max microplate spectrophotometer (Molecular Devices).

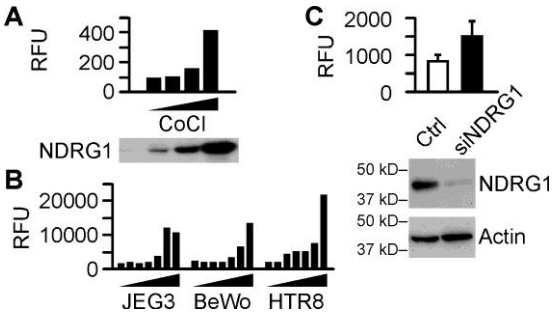

Fig. 6: The influence of NDRG1 silencing on apoptosis in trophoblast cell lines. (A) CoCl induces apoptosis in JEG3 cells. Caspase 3 activity, determined using a fluorescent assay and presented as relative fluorescence units (RFU), normalized to cellular protein, corresponds to NDRG1 expression. (B) UV light induces apoptosis in relevant cell lines. UV radiation at an energy range of 0-10.0 mj/CM2 was produced using a UV crosslinker. Caspase 3 activity determined as in panel A. (C) siRNA-mediated silencing of NDRG1 enhances apoptosis in JEG3 cells. Caspase 3 activity determined as in panel A. Western blot shown below the panel, as control for silencing efficiency.

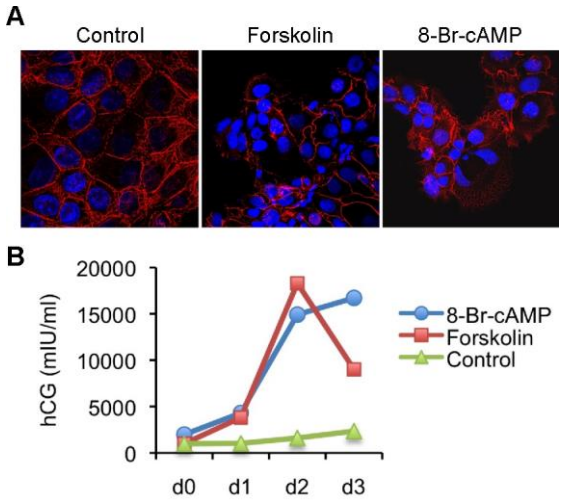

Fig 7: The effect of forskolin or 8-Br-cAMP on syncytial formation in BeWo cells. (A) Cell membrane (red) was detected using anti-desmosomal Ab, and nuclei (blue) were visualized with Hoechst 33342. (B) hCG release from cells exposed to PKA activators.

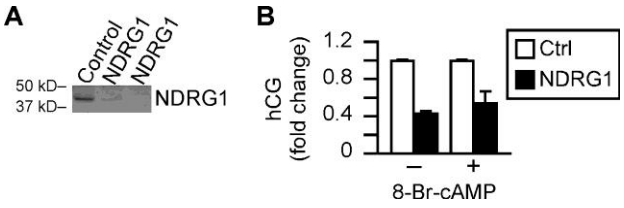

Fig. 8: Silencing of NDRG1 attenuates differentiation of BeWo cells. (A) Two different NDRG1 siRNA constructs were effective in silencing NDRG1 expression. (B) The effect of NDRG1 silencing on hCG release in BeWo cells, in the absence or presence of 8-Br-cAMP (0.5 mM for 24h).

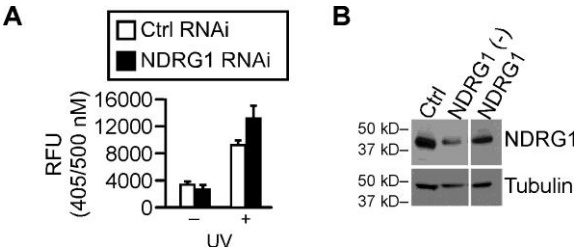

Fig. 9: Silencing of NDRG1 in JEG3 potentiates UV light-induced apoptosis. (A) The rate of apoptosis was determined using the caspase 3 assay, as described earlier. (B) Western blot control for NDRG1 silencing (second lane) and rescue (third lane).

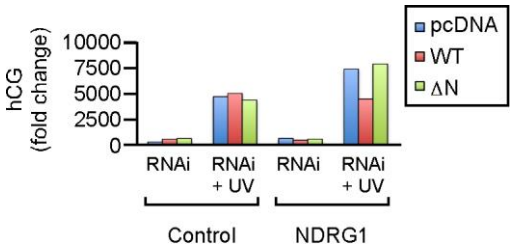

Fig.10: The impact of wild type or NDRG1deltaN on UV-induced apoptosis in JEG3 cells. Apoptosis was induced by UV light (6 mj/sq cm), and confirmed using a caspase 3 assay.

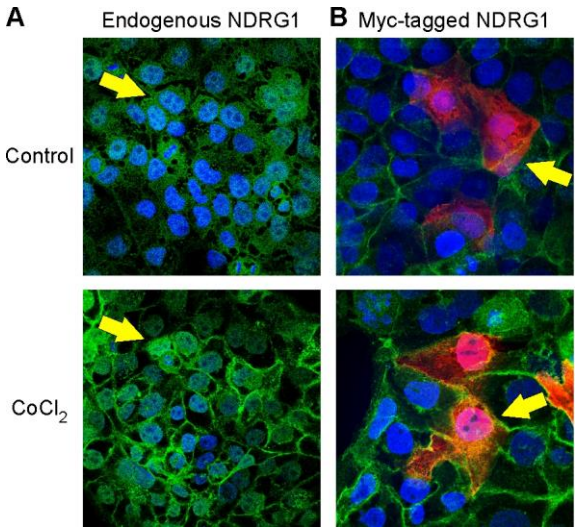

Fig.11: Immunofluorescence analyzed using confocal microscope, showing that CoCl enhances the translocation of wt or recombinant NDRG1 into the nucleus and cell membrane of JEG3 cells. (A) Endogenous NDRG1 (green fluorescence), detected using an anti-NDRG1 antibody. Arrows show nuclei (blue stain using Hoechst 3342) before and after CoCl. (B) Exogenous, recombinant NDRG1-myc (red fluorescence), detected using an anti-myc antibody. Arrows show nuclei before and after CoCl. Cell membrane (green) detected using E-cadherin antibody. Arrows show nuclei (blue stain using Hoechst 33342) before and after CoCl.

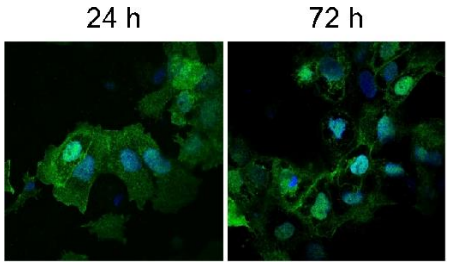

Fig. 12: Hypoxia enhances nuclear localization of NDRG1 in primary human trophoblasts. Trophoblasts were cultured in standard (FiO_2=20%) or hypoxic (FiO_2=1%) atmosphere for 24-72 h, and NDRG1 detected using a rabbit anti-NDRG1 polyclonal antibody (Zymed) followed by cell incubation with Alexafluor488-linked goat anti-rabbit antibody. Nuclei (blue) were visualized with Hoechst 33342. The images were captured with an Olympus AX70 Provis fluorescence microscope, equipped with Olympus Fluoview 1000 confocal imaging system.

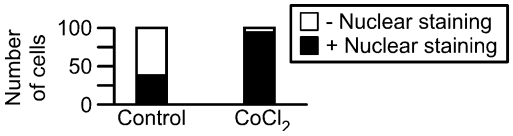

Fig. 13: Quantification of nuclear staining by NDRG1, performed by counting 100 cells in five different slides. The difference was significant at $p < 0.05$.

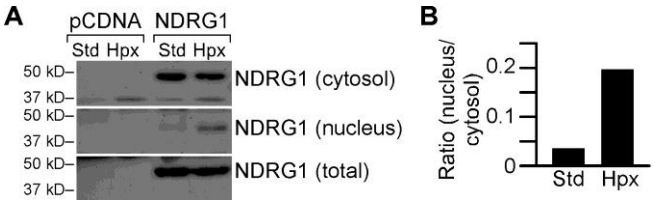

Fig. 14: The effect of hypoxia on expression of NDRG1 in isolated nuclei. (A) Nuclei and cytoplasm were isolated, and NDRG1 levels were determined by western immunoblotting. Tubulin (expressed in cytoplasm) and Lamin A/C (expressed in nuclei) served as control. (B) Analysis of nuclei/cytoplasm NDRG1 ratio.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American

_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X____ No_____

If yes, please describe your plans:

I expect to publish at least one major article that describes the functional domains within NDRG1. I also expect that some of these functional domains will be studied in vivo (this component of our research was supported by this grant, but not an integral part of the one year project). Additional activities of NDRG1, based on research advanced by this grant, will be published as well.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The research supported by this one-year project centered on a structure-function analysis of a protein. While the majority of the goals were achieved, the full completion of the aims and the attainment of benefits from the described discoveries await additional research. These

discoveries, once fully analyzed, may be translated to improved ability to diagnose placental diseases or even alter their course.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

At the present time our major discovery is the definition of NDRG1's function during placental injury, demonstrating that NDRG1 mitigates the injury of human placental trophoblasts in response to common placental injuries such as hypoxia. The translation of these discoveries to prevention, diagnosis or treatment of disease awaits further research (see response to question #21).

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Yoel Sadovsky, MD
Scientific Director, Magee-Womens Research Institute, Professor of OBGYN and Reproductive Sciences, and Microbiology and Molecular Genetics

EDUCATION/TRAINING INSTITUTION	DEGREE	YEAR(s)	FIELD OF STUDY
Hebrew University, Jerusalem Israel	BS	1981	Biomedical sciences
Hebrew University, Jerusalem Israel	MD	1985	Medicine
Washington University, St. Louis		1986-90	Resident, OBGYN
University of California, San Francisco	Fellow	1990-92	Maternal-Fetal Medicine
University of California, San Francisco	Postdoctorate	1992-93	Gene regulation

A. Professional experience and appointments

Washington University in St. Louis

1993	Assist Professor, Maternal-Fetal Medicine, OBGYN
1997-2007	Joint Appointment: Department of Cell Biology and Physiology,
1998-2007	Member, Division of Biology and Biomedical Sciences
1999	Associate Professor (tenure), OBGYN, Cell Biology and Physiology
1999-2007	Director, Division of Maternal-Fetal Medicine and Ultrasound
2004-2007	Professor (tenure), OBGYN, and Cell Biology and Physiology

Magee-Womens Research Institute, University of Pittsburgh

2007-Present	Scientific Director, Magee-Womens Research Institute
2007-Present	Professor (tenure) and Vice Chair, OBGYN and Reproductive Sciences
2007-Present	Elsie Hilliard Hillman Chair of Women's Health Research
2009-Present	Professor of Microbiology and Molecular Genetics

Honors and awards

1983	Dean's Award, Hebrew University, Israel
1986	"Best Intern" award. Hadassah Medical Center, Jerusalem
1995, 1997-2000, 2002, 2004, 2005	Excellence in Teaching Award, Department of OBGYN, Washington University
1996-1997	Berlex Foundation Scholar Award
1997	Excellence in Teaching Award, Assoc of Professors of Gyn/Ob
1999, 2000, 2003	Teacher of the Year Award, Washington University
2002-2006	NIH-NICHD Maternal and Child Health Research Study Section
2005-2006	Chair, NICHD Obstetrics, Mat-Fetal Biology Research Study Section
2004	Alpha Omega Alpha Honor Society
2004	Society for Gynecologic Investigation President's Achievement Award
2005-2010	Steering Committee Chair, NICHD Genomic and Proteomic Network for Preterm Birth Research
2007-2008	President, Perinatal Research Society
2009-Present	Council member, Society for Gynecologic Investigation
2009-2013	NIH-NICHD National Advisory Child Health and Human Development Council Member

C. Selected recent publications (from a list of 104)

1. Yan X, Mouillet JF, Ou Q, Sadovsky Y. A novel domain within the DEAD-box protein DP103 is essential for transcriptional repression and helicase activity. *Mol Cell Biol* 2003;28:414-23.
2. Budhraja V, Spitznagel E, Schaiff WT, Sadovsky Y. Incorporation of gene-specific variability improves expression analysis using high-density DNA microarrays. *BMC Biol* 2003;1:1.
3. Mouillet JF, Sonnenberg-Hirche C, Yan X, Sadovsky Y. p300 regulates the synergy of SF-1 and Egr-1 in activating LHβ gene. *J Biol Chem* 2004;279:7832-9.
4. Chen BS, Nelson DM, Sadovsky Y. NDRG1 modulates the response of term human trophoblasts to hypoxic injury. *J Biol Chem* 2006;281:2764-72.
5. Schaiff WT, Knapp, Jr. FF, Barak Y, Biron-Shental T, Nelson DM, Sadovsky Y. Ligand-activated PPARγ alters placental morphology and placental fatty acid uptake in mice. *Endocrinology* 2007;148:3625-34.
6. Rimon E, Chen BS, Shanks AL, Nelson DM, Sadovsky Y. Hypoxia in human trophoblasts stimulates the expression and secretion of connective tissue growth factor. *Endocrinology* 2008;149:2952-8. PMC2408813
7. Tomlinson TM, Garbow JR, Anderson JR, Engelbach JA, Nelson DM, Sadovsky Y. Magnetic resonance imaging of hypoxic injury to the murine placenta. Am J Physiol Regul Integr Comp Physiol 2010,298:R312-9, PMC pending.
8. Mouillet JF, Chu T, Nelson DM, Mishima T, Sadovsky Y. miR-205 silences MED1 in hypoxic primary human trophoblasts. *FASEB J*, Published online Jan 11 2010. PMC pending.

D. Research Support

Ongoing

NIH/NIEHS R01 ES11597 (PI: Sadovsky)	9/01–5/12
Regulation of trophoblast differentiation and function	
NIH/NICHD R01 HD045675 (PI: Sadovsky)	7/04–4/09
Placental injury and adaptation	NCE 4/30/10
NIH/NICHD K12 HD063087-01 (PI: Hogge, RD: Sadovsky)	9/09-8/14
Magee-Womens Basic and Translational Reproductive Health Training Program	
Penn. Dept. of Health Research Formula (PI: Mouillet; Co-I: Sadovsky)	1/10–12/10
The role of microRNA-424 in differentiation of placental trophoblasts	

Completed

NIH/NIAID NO1-AI25496 (PI: Bloomberg; Collab: Sadovsky)	5/04–2/07
ICAC: Immunologic approaches to reduce asthma	
March of Dimes 21-FY04-177 (PI: Muglia; Collab: Sadovsky)	3/05–2/08
Genetic analysis of human premature birth	
NIH/NICHD R21 HD053878 (PI: Sadovsky)	8/06–7/08
The role of microRNA in placental differentiation	
NIH/NICHD R01 HD29190 (PI: Nelson; Co-I: Sadovsky)	4/07–3/09
Human placental trophoblast response to hypoxia	
Penn. Dept. of Health Research Formula Funds (PI: Sadovsky)	1/09–12/09
Analysis of functional domains within NDRG1	