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Gene Expression in the Mouse Placenta: Developmental and Stress Responses

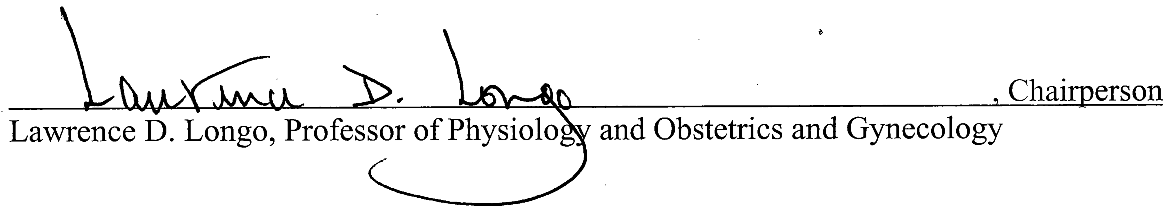
by

Ciprian P. Gheorghe

A Thesis submitted in partial satisfaction
of the requirements for the degree of
Doctor of Philosophy in Physiology

September 2006

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.



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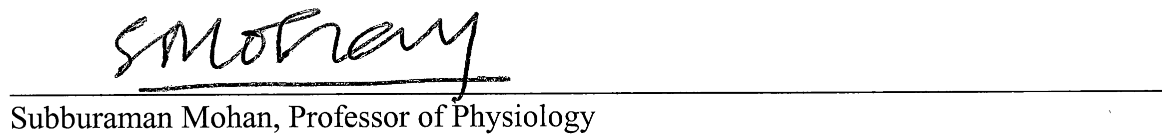

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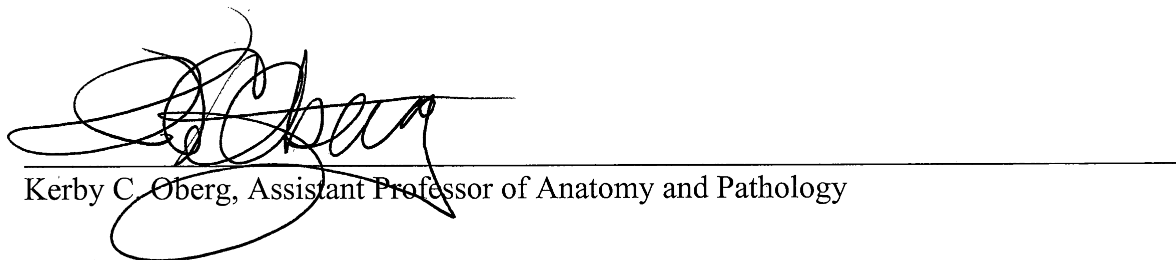

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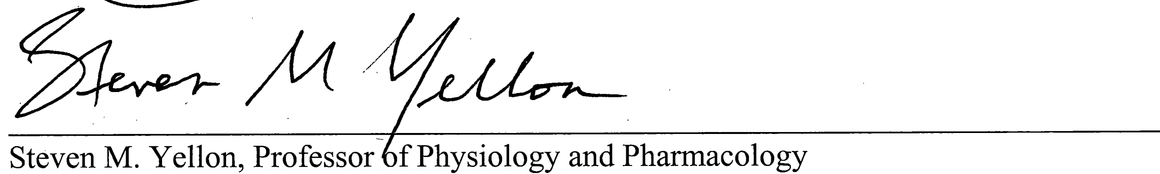

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Lawrence D. Longo for his unwavering support, help and belief in me. He has taught me how to become a scientist and how to pursue important ideas. I will forever be indebted to him for his guidance.

I would also like to thank the other members of my committee: Dr. Subburaman Mohan, Dr. Kerby Oberg and Dr. Steve Yellon for their advice, encouragement and guidance.

I would like to thank my wife, Stacy Gheorghe, for her patience, assistance and for her technical help.

I would like to thank my parents, Paul and Ligia Gheorghe for passing on to me their love of learning and discovery. They have always encouraged and helped me. I would also like to thank the rest of my family, especially my uncle Viorel and aunt Camelia for opening their home to me and always being available to help. My in-laws Dawn Temple and Philip Lang have also been extremely supportive throughout this arduous process.

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ABSTRACT OF THE DISSERTATION

Gene Expression in the Mouse Placenta: Developmental and Stress Responses

by

Ciprian P. Gheorghe
Doctor of Philosophy, Graduate Program in Physiology

Loma Linda University, September 2006

Dr. Lawrence Longo, Chairperson

Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. Placental failure and placental pathology contributes to both morbidity and mortality of the fetus. We sought to understand normal placental development and also placental responses to stress using oligonucleotide microarray technology. To examine genetic aspects of normal placental development, we investigated gene expression patterns in the murine placenta at embryonic day 10.5 (E10.5), E12.5, E15.5, and E17.5. Hypoxia has been identified as a major stressor in placental and fetal development. In order to comprehend more completely hypoxic stress responses we sought to measure gene expression changes in the mouse placenta in response to 48 hours of hypoxia from E15.5 to 17.5. Also, maternal protein restriction has been shown to have deleterious effects on placental development and has long term consequences for the progeny. In order to examine stress responses to maternal protein restriction, we sought to measure gene expression changes in the mouse placenta in response to 7 days of protein restriction from E10.5 to E17.5. Angiogenesis and fatty acid metabolism and transport related genes were up-regulated at E10.5, while genes involved in hormonal control and ribosomal proteins were up-regulated at E12.5. Genes involved

in the cell cycle and RNA metabolism were up-regulated at E12.5, while genes involved in cellular transport were up-regulated at E15.5. Genes related to cell cycle control, genes expressed in the nucleus and involved in RNA metabolism were up-regulated at E17.5. We observed that hypoxia up-regulated proteins involved in reactive oxygen species metabolism and DNA methylation enzymes. Also, apoptosis related genes were up-regulated. In response to protein deprivation negative regulators of cell growth and metabolism in conjunction with genes involved in epigenesis were up-regulated. The presence of epigenetic regulating proteins suggests that hypoxia and protein deprivation may contribute to growth restriction and long-term epigenetic changes in stressed tissues and organs.

Chapter 1

Placental Development

Introduction

The placenta is the organ associated with fetal development that performs various functions: respiratory gas and nutrient exchange, immune modulation, hormonal control and metabolism. The placenta protects the fetus from the maternal immune system by modulating and inhibiting maternal rejection of the fetus that is an allograft, because it expresses paternal epitopes that are normally recognized as foreign. Even when mothers were purposely sensitized with paternal homografts, the placenta was able to protect the fetuses from any immune toxicity [1]. By providing a large surface area, the placenta promotes oxygen exchange, nutrient trafficking, and toxic waste removal. In the human, the trophoblast achieves an exchange surface area of 12.5 sq meters. The mean trophoblast thickness is 4.1 μm and the diffusion distance is 4.8 μm [2]. Through the secretion of hormones, the placenta modulates the endocrine and metabolic milieu of the mother in order to benefit the fetus and allow it to thrive. Placental lactogen and chorionic gonadotrophin are the two main hormones of pregnancy, and their roles are to modulate maternal and fetal metabolism and growth.

Proper development and growth of the placenta is essential for the development of the fetus. Stress, or genetic defects that lead to placental dysfunction, cause fetal distress and lead to intra-uterine growth restriction (IUGR). In diseases such as pre-eclampsia, placental dysfunction can lead to severe maternal morbidity and to mortality. The genetic basis of placental development has been poorly understood. Recently, the mouse has emerged as a useful model for placental development. Even though the morphology of

the mouse placenta differs from the human, similar cell lineages and genetic mechanisms appear to govern placental development in both species. Both placentae are classified as chorio-allantoic, and have a haemochorial interface between the mother and the fetus. In particular, they differ in the arrangement of the branching of the maternal-fetal interface. The human placenta is considered of the villous type because of the tree or branch-like pattern and the blunt ends of the villi. The mouse placenta has a much more interdigitated pattern that results in a maze-like structure termed the labyrinth [3]. Despite these morphological differences, extensive studies have shown that the mouse and human placenta have similar cell lineages that not only perform the same biological function but also share similar gene markers [4].

Hypoxia and nutritional deprivation have been shown to be important stressors that adversely affect fetal development. High altitude studies in our laboratory have demonstrated the profound effects of chronic hypoxic exposure on the mother and the fetus. The physiological adaptations to long-term hypoxia for both the mother and fetus are summarized in figures 2 and 3. Besides these general physiological parameters, fetal hypoxia affects vascular contractility, hormonal responses, and brain development [5-7].

Fetal hypoxia can be a consequence of several conditions: maternal lung disease, maternal heart disease, maternal anemia, living at high altitude, maternal malnutrition, maternal smoking, or primary placental dysfunction. Placentas from pregnancies affected by intra-uterine growth restriction display impaired vasculogenesis, altered expression of nutrient transporters, and altered trophoblast function [8-10]. Studies in our laboratory have shown that in the sheep, in response to long-term hypoxia, placental morphology and placental vascular structure were altered significantly [11, 12].

In an effort to understand more completely the mechanisms underlying these changes, we decided to examine gene expression during normal development, and also to identify changes that occur in response to hypoxia and maternal nutritional protein deprivation. We chose the mouse as a model because of its genetic similarities to the human, and the ease of manipulation and analysis. The entire genomic sequence for the mouse is available making genetic analysis easier to undertake. Also, the mouse has a relatively short gestation period (20 to 21 days depending on the strain), and high fertility. We examined gene expression throughout normal placental development, and also in response to hypoxia and maternal nutritional protein deprivation.

Mouse placental development

Cell lineages

The mature murine placenta derives from two disctinct cell lineages. The outer epithelium is derived from the trophoblast cell lineage, while the vascular and mesenchemal tissues are derived from the embryonic mesoderm. The placental structure is established by the fusion of the allantois with the ectoplacental cone at embryonic day 8.5 (E8.5). By E10 the distinct layers of the mature placenta are established, and the vascular network is formed. The throphoblast layer is comprised of three distinct cell types and layers. The innermost labyrinth is formed at E9 and is a highly folded and invaginated layer which facilitates respiratory gas and nutrient exchange. The intermediate layer is the spongiotrophoblast and is formed from the ectoplacental cone.

The outermost layer is formed by trophoblast giant cells. These are polyploidy multinucleated cells that invade the blood vessels in the maternal decidua, and modulate both the increase in blood flow to the placenta and the down-regulation of the maternal immune response.

Early changes

It is in the blastocyst that the trophoblast cell lineage arises prior to implantation. Cells of the blastocyst differentiate either as inner cell mass (ICM) or trophoblast [13]. Two factors appear to be crucial in deciding cell fates: cell positioning and the presence of the transcription factor Oct4. Outer cells will differentiate into trophoblast, and downregulation of the Oct4 transcription factor is necessary for ICM development. Oct4 null mutants do not develop an ICM, and rather the entire blastocyst develops into trophoblast [14]. The ICM also directs trophoblast development through the paracrine expression of several growth factors. For instance, fibroblast growth factor 4 (Fgf4) has been shown to be required for trophoblast development [15]. Fgf4 null-mutants arrest at the blastocyst stage, and in contrast cell culture blastocysts will develop into trophoblast if Fgf4 is included in the medium [16]. Although Fgf4 is expressed in the ICM, FGF Receptor expression is restricted to the outer trophoblast layers. In turn, Fgf4 appears to exert its' effects through two transcription factors: Cdx and Eomes [17].

Allantoic fusion and labyrinth formation

The fusion of the allantois with the ectoplacental cone (EPC) is mediated by a number of molecules. Several molecules are necessary for normal allantoic and chorionic

development. Mutations in Brachyury [18], DNA methyl transferase [19], and Lim1 [20] produce abnormal allantois structures. Err2 gene mutations result in the absence of chorionic trophoblast cells [21]. Other molecules appear to be involved in the fusion of the two structures. Vcam1 and alpha-4-integrin are binding partner molecules expressed at the allananto-chorionic fusion site [22]. Vcam1 is expressed at the tip of the allantois, while alpha-4-integrin is expressed at on the chorionic plate. Mutations in either of these molecules results in the failure of fusion, despite both structures appearing grossly normal [23].

Following successful alanto-chorionic fusion, the labyrinth forms through complex molecular and morphological mechanisms. Studies in null mutant mice have revealed that several molecules appear to be crucial to successful labyrinth formation. For example, mutations in Dlx3 [24], Esx1 [25], Fgfr2 [26], JunB [27], Rxr-a [28], Hgf [29], c-Met [30], Integrin $\alpha5$ [31], Wnt2 [32] all lead to embryonic lethality due to deficiencies in labyrinth formation. Two distinct types of deficiencies lead to labyrinth failure: trophoblast defects and vascular defects. The majority of the defects appear to be trophoblast based, while Esx1 [33] , Arnt [25] and Tcfeb deficient mice [34] demonstrate defects in vascular development. The role in normal placental development of these molecules remains unclear, however the common mechanism of lethality in the null mutants points to their importance.

Trophoblast Giant Cells

The development of trophoblast giant cells (TGC) is a more thoroughly understood process. Giant cells are polyploid cells as a result of endoreplication (i.e.,

DNA replication occurs without intervening mitoses) [35]. TGC are quite invasive and can be found deep within the deciduas. They secrete several pro-angiogenic and vasoactive molecules that transform the maternal circulation at the decidual-placental interface. They also produce immune modulatory molecules that regulate lymphocyte activation. TGC differentiation is regulated by several transcription factors of the Basic helix loop helix family (bHLH). These bHLH transcription factors contain a dimerization domain which appears to be crucial in their biological activity [36], and can either inhibit or induce TGC differentiation. Mash2 and mSna promote TGC differentiation [37, 38], while Hand1 inhibits it [39]. These genes interact in a complex manner and their actions are modulated by their binding partners Alf1 and Itf2 [40].

Parallels to human placental development

The gross structure of the human and mouse placenta differs significantly, however to a great extent the cell lineages are conserved between the two species. Also, the molecular determinants of placental development appear to have been conserved. In great part due to the development of mouse knockout models, numerous genes have been implicated in placental development.

It is hoped that though studies of the development of mouse placenta several human disorders can be better understood and treated. These include early pregnancy loss, certain forms of intra-uterine growth restriction (IUGR) and pre-eclampsia. Each of these disorders appears to affect a different aspect of placental development: pre-eclampsia affects extra-villous trophoblast cell invasion, while IUGR affects the placental

vasculature and chorionic villi. These distinct defects resemble the various phenotypes of the mouse mutants. The extra-villous trophoblast, involved in pre-eclampsia in the human, corresponds to the trophoblast giant cells in the mouse, while the chorionic villi, involved in IUGR and pregnancy loss, correspond to the labyrinthine structures. In the mouse defects of those two regions are cause by distinct genes and pathways, and it would be expected that similar relationships exist for human pathologies.

Placental dysfunction also is caused by external stressors, and the present studies have identified several crucial genetic and signaling pathways that are triggered by hypoxic stress and protein deprivation. While the conserved nature of these responses between the mouse and the human remain to be confirmed, our results provide intriguing leads into understanding and addressing the consequences and potentially deleterious long-term effects of placental stress.

References

1. Lanman, J.T. and L. Herod. Homograft immunity in pregnancy. The placental transfer of cytotoxic antibody in rabbits. J Exp Med, 1965; 122: 579-86.

2. Myatt, L. Placental adaptive responses and fetal programming. J Physiol, 2006; 572: 25-30.

3. Kirby, D.R. and S. Bradbury. The hemo-chorial mouse placenta. Anat Rec, 1965; 152: 279-81.

4. Georgiades, P., A.C. Ferguson-Smith, and G.J. Burton. Comparative developmental anatomy of the murine and human definitive placentae. Placenta, 2002; 23: 3-19.

5. Longo, L.D. and W.J. Pearce. Fetal and newborn cerebral vascular responses and adaptations to hypoxia. Semin Perinatol, 1991; 15: 49-57.

6. Alonso, J.G., T. Okai, L.D. Longo, and R.D. Gilbert. Cardiac function during long-term hypoxemia in fetal sheep. Am J Physiol, 1989; 257: H581-9.

7. Xiao, D., X. Huang, S. Bae, C.A. Ducsay, L.D. Longo, and L. Zhang. Cortisol-mediated regulation of uterine artery contractility: effect of chronic hypoxia. Am J Physiol Heart Circ Physiol, 2004; 286: H716-22.

8. Nelson, D.M., S.D. Smith, T.C. Furesz, Y. Sadovsky, V. Ganapathy, C.A. Parvin, and C.H. Smith. Hypoxia reduces expression and function of system A amino acid transporters in cultured term human trophoblasts. Am J Physiol Cell Physiol, 2003; 284: C310-5.

9. Alfaidy, N., S. Gupta, C. DeMarco, I. Caniggia, and J.R. Challis. Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications. J Clin Endocrinol Metab, 2002; 87: 4797-805.

10. Kingdom, J.C. and P. Kaufmann. Oxygen and placental villous development: origins of fetal hypoxia. Placenta, 1997; 18: 613-21; discussion 23-6.

11. Penninga, L. and L.D. Longo. Ovine placentome morphology: effect of high altitude, long-term hypoxia. Placenta, 1998; 19: 187-93.

12. Krebs, C., L.D. Longo, and R. Leiser. Term ovine placental vasculature: comparison of sea level and high altitude conditions by corrosion cast and histomorphometry. Placenta, 1997; 18: 43-51.

13. Copp, A.J. Interaction between inner cell mass and trophectoderm of the mouse blastocyst. II. The fate of the polar trophectoderm. J Embryol Exp Morphol, 1979; 51: 109-20.

14. Nichols, J., B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Scholer, and A. Smith. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell, 1998; 95: 379-91.

15. Feldman, B., W. Poueymirou, V.E. Papaioannou, T.M. DeChiara, and M. Goldfarb. Requirement of FGF-4 for postimplantation mouse development. Science, 1995; 267: 246-9.

16. Wilder, P.J., D. Kelly, K. Brigman, C.L. Peterson, T. Nowling, Q.S. Gao, R.D. McComb, M.R. Capecchi, and A. Rizzino. Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. Dev Biol, 1997; 192: 614-29.

17. Lohnes, D. The Cdx1 homeodomain protein: an integrator of posterior signaling in the mouse. Bioessays, 2003; 25: 971-80.

18. Rashbass, P., L.A. Cooke, B.G. Herrmann, and R.S. Beddington. A cell autonomous function of Brachyury in T/T embryonic stem cell chimaeras. Nature, 1991; 353: 348-51.

19. Li, E., T.H. Bestor, and R. Jaenisch. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell, 1992; 69: 915-26.

20. Shawlot, W. and R.R. Behringer. Requirement for Lim1 in head-organizer function. Nature, 1995; 374: 425-30.

21. Luo, J., R. Sladek, J.A. Bader, A. Matthyssen, J. Rossant, and V. Giguere. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. Nature, 1997; 388: 778-82.

22. Yang, J.T., H. Rayburn, and R.O. Hynes. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development, 1995; 121: 549-60.

23. Gurtner, G.C., V. Davis, H. Li, M.J. McCoy, A. Sharpe, and M.I. Cybulsky. Targeted disruption of the murine VCAM1 gene: essential role of VCAM-1 in chorioallantoic fusion and placentation. Genes Dev, 1995; 9: 1-14.

24. Morasso, M.I., A. Grinberg, G. Robinson, T.D. Sargent, and K.A. Mahon. Placental failure in mice lacking the homeobox gene Dlx3. Proc Natl Acad Sci U S A, 1999; 96: 162-7.

25. Adelman, D.M., M. Gertsenstein, A. Nagy, M.C. Simon, and E. Maltepe. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev, 2000; 14: 3191-203.

26. Arman, E., R. Haffner-Krausz, Y. Chen, J.K. Heath, and P. Lonai. Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci U S A, 1998; 95: 5082-7.

27. Schorpp-Kistner, M., Z.Q. Wang, P. Angel, and E.F. Wagner. JunB is essential for mammalian placentation. Embo J, 1999; 18: 934-48.

28. Wendling, O., P. Chambon, and M. Mark. Retinoid X receptors are essential for early mouse development and placentogenesis. Proc Natl Acad Sci U S A, 1999; 96: 547-51.

29. Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda, and N. Kitamura. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature, 1995; 373: 702-5.

30. Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier. Scatter factor/hepatocyte growth factor is essential for liver development. Nature, 1995; 373: 699-702.

31. Goh, K.L., J.T. Yang, and R.O. Hynes. Mesodermal defects and cranial neural crest apoptosis in alpha5 integrin-null embryos. Development, 1997; 124: 4309-19.

32. Monkley, S.J., S.J. Delaney, D.J. Pennisi, J.H. Christiansen, and B.J. Wainwright. Targeted disruption of the Wnt2 gene results in placentation defects. Development, 1996; 122: 3343-53.

33. Li, Y. and R.R. Behringer. Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. Nat Genet, 1998; 20: 309-11.

34. Steingrimsson, E., L. Tessarollo, S.W. Reid, N.A. Jenkins, and N.G. Copeland. The bHLH-Zip transcription factor Tfeb is essential for placental vascularization. Development, 1998; 125: 4607-16.

35. Zybina, E.V. and T.G. Zybina. Polytene chromosomes in mammalian cells. Int Rev Cytol, 1996; 165: 53-119.

36. Firulli, A.B. A HANDful of questions: the molecular biology of the heart and neural crest derivatives (HAND)-subclass of basic helix-loop-helix transcription factors. Gene, 2003; 312: 27-40.

37. Nakayama, H., I.C. Scott, and J.C. Cross. The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor. Dev Biol, 1998; 199: 150-63.

38. Guillemot, F., A. Nagy, A. Auerbach, J. Rossant, and A.L. Joyner. Essential role of Mash-2 in extraembryonic development. Nature, 1994; 371: 333-6.

39. Riley, P., L. Anson-Cartwright, and J.C. Cross. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet, 1998; 18: 271-5.

40. Scott, I.C., L. Anson-Cartwright, P. Riley, D. Reda, and J.C. Cross. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. Mol Cell Biol, 2000; 20: 530-41.

Chapter 2

Gene expression patterns in the developing murine placenta

Ciprian Gheorghe[1], Subburaman Mohan, PhD,[2], and Lawrence D. Longo MD[1].

Running Head: Gene expression in the murine placenta

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Supported, in part by USPHS grant HD-03807 to LDL and the Society for Gynecological Investigation Medical Student Grant to CPG.

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Abstract

OBJECTIVE: Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. To examine genetic aspects of placental development, we investigated gene expression patterns in the murine placenta at embryonic day 10.5 (E10.5), E12.5, E15.5, and E17.5.

METHODS: By use of the Affymetrix MU74A array, we measured expression levels for 12,473 probe sets. Using pairwise analysis we selected 622 probe sets, corresponding to 599 genes, that were up- or down-regulated by more than four-fold between time points E10.5 and E12.5, E12.5 and E15.5, E15.5 and E17.5. We analyzed and functionally annotated those genes regulated during development.

RESULTS: In comparing E10.5 to E12.5 we found that angiogenesis and fatty acid metabolism and transport related genes were up-regulated at E10.5, while genes involved in hormonal control and ribosomal proteins were up-regulated at E12.5. When comparing E12.5 to E15.5 we noted that genes involved in the cell cycle and RNA metabolism were strongly up-regulated at E12.5, while genes involved in cellular transport were up-regulated at E15.5. Finally, when comparing E15.5 to E17.5, we found genes related to cell cycle control, genes expressed in the nucleus and involved in RNA metabolism were up-regulated at E17.5.

CONCLUSIONS: Microarray analysis has allowed us to describe gene expression patterns and profiles in the developing mouse placenta. Further analysis has demonstrated that several functional classes are up- and down-regulated at specific time points in placental development. These changes may have significant implications for placental development in the human.

Introduction

Successful placental development is crucial for optimal growth, maturation, and survival of the embryo/fetus. This is evidenced by the numerous lethal embryonic null-mutants secondary to placental failure. Placental cell lineages derive from trophoectoderm precursors. The mural trophoectoderm differentiates into primary trophoblast giant cells while the polar trophoectoderm gives rise to the extraembryonic ectoderm and the ectoplacental cone. The extraembryonic ectoderm forms the chorion which fuses with the allantois, an outgrowth of extraembryonic mesoderm, at around E8 to form the labyrinthine layer of the placenta. The spongiotrophoblast layer of the murine placenta forms from ectoplacental precursor cells and forms the middle layer of the placenta (also known as the junctional zone). The outer layer of the placenta is the trophoblast giant cell layer. In addition to the primary trophoblast cells derived from the mural trophoectoderm, are secondary trophoblast giant cells derived from the spongiotrophoblast. Later in placental development, around E12.5, glycogen trophoblast cells appear in the spongiotrophoblast layer. Although their function is unclear, they do express several important gene products and migrate into the decidua later in pregnancy. Several reviews have detailed placental cell lineages and some of the genes involved in their differentiation [1, 2].

Recent studies are beginning to reveal the fundamental mechanisms underlying placental development [3]. The number of genes known to be involved in this process have increased greatly, partly due to the discovery of numerous lethal embryonic null mutants secondary to placental failure[4-8]. To understand this process at a more fundamental level, by use of DNA microarray technology, we examined gene expression

patterns in the developing murine placenta at days E10.5, E12.5, E15.5, and E17.5. Numerous genes are required for proper placental development. For instance, the disruption of many genes, including growth factors, transcription factors, extracellular matrix proteins, and proteins involved in cell signaling leads to embryonic lethality secondary to placental failure [9]. In differentiating human trophoblast *in-vitro*, several gene classes are strongly up- and down-regulated [10]. Another study compared differentially expressed genes between the murine placenta and embryo at E12.5 [11]. Those reports demonstrated the dynamic involvement of numerous gene classes in placental development, and that microarrays can be powerful tools in elucidating these processes. To our knowledge, this is the first examination of gene expression in the murine placenta throughout development using microarray analysis. In the present study, we tested the hypothesis that from E10.5 until E17.5 in the mouse placenta, numerous placental genes are up- or down- regulated to a significant degree, and that specific functional groups of genes are regulated at different developmental stages.

Materials and Methods

Animals

Eight-week old FVB/NJ male and female mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed at the Animal Research Facility, Loma Linda University, Loma Linda, CA under conditions of 14 h light, 10 h darkness, ambient temperature of 20° C, and relative humidity of 30-60%. All experimental protocols were

in compliance with the Animal Welfare Act, and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Breeding and tissue collection:

Animals were bred overnight and the morning when the vaginal plug was observed was considered E 0.5 of pregnancy. Pregnant females were sacrificed by CO_2 asphyxiation at E10.5, E12.5, E15.5, and E17.5. The uterus was removed rapidly and placed in a petri dish containing RNALater solution (Ambion, Austin, TX). Placentas were isolated under a dissection microscope, and stored at 4°C in microcentrifuge tubes. We determined the developmental stages of the embryos by visual inspection according to a modified Theiler staging system [12]. Details of the staging system are available online at http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml. Placentas from each animal, and six animals from each time point were pooled, total RNA was isolated using the TRIZOL reagent (Life Technologies, Rockville, MD), and the total RNA was stored at -80 °C.

Probe preparation, microarray hybridization, and data analysis:

The RNA was processed for use on the Affymetrix MU74A array (Affymetrix, Santa Clara, CA), according to the manufacturer's instructions. We pooled total RNA from six mice at each time point. Briefly, 5 μg of total RNA was reverse transcribed to double stranded cDNA (Superscript II kit, Life Technologies). The double stranded cDNA was used in an *in-vitro* transcription reaction to generate biotynilated cRNA. This cRNA was purified, fragmented, and hybridized to the Affymetrix chip. Washes and staining were performed in an Affymetrix Gene Chip Fluidics station 400. The Affymetrix arrays were

scanned using a Gene Array Scanner (Hewlett Packard, Austin, TX), and processed at the Microarray Facility, University of California Irvine, (Irvine, CA). Data analysis was performed using the GeneSpring software (Silicon Genetics, Santa Clara, CA), and hybridization intensities were normalized to the median value for each array. We performed pair-wise analysis between E10.5 and E12.5, E12.5 and E15.5 and E15.5 and E17.5. Genes up- or down-regulated by four-fold between these time-points were considered developmentally regulated, and were selected for further analysis. Genes were also filtered based on Affymetrix flags. Those having absent or marginal flags at the two time points being compared were discarded from the analysis. We repeated the hybridization at E10.5 with a different RNA sample to determine the amount of variation present. In this repeat experiment, only 7 out of 12,473 genes varied in excess of four-fold, and those that varied had an absent Affymetrix call in one of the two samples, suggesting that poorhybridization was responsible for the variability.

The genes were assigned to functional classes based on the GO database (http://www.geneontology.org/GO.annotation.html). Significantly over-represented GO categories in the gene sets were analyzed using the Gene Ontology Tree Machine (http://genereg.ornl.gov/gotm/) and the DAVID and EASE softwares (http://david.niaid.nih.gov/david/).

Real Time PCR

In an effort to validate the results of the microarray analysis, we randomly chose several genes that were shown to be developmentally regulated for analysis using real time PCR. We retrieved verified real-time PCR primers from Primer Bank

(http://pga.mgh.harvard.edu/primerbank/index.html) ensuring that they were intron-spanning and they corresponded to the genes present on our microarray. The primers were synthesized by Integrated DNA technologies (Coralville, CA). The primer sequences selected were:

Growth Hormone Releasing Hormone:

Forward: 5'- GGT GCT CTT TGT GAT CCT CAT C -3'

Reverse: 5'- GTT TCC TGT AGT TGG TGG TGA AG -3'

Vanin 1:

Forward: 5'- CTT TCC TCG CGG CTG TTT AC -3'

Reverse: 5'- CCT CCA GGT ATG GGT AGA TCG T -3'

Insulin-Like Growth Factor Binding Protein 2:

Forward: 5'- CAG ACG CTA CGC TGC TAT CC -3'

Reverse: 5'- CCC TCA GAG TGG TCG TCA TCA -3'

Placenta Specific Homeobox Gene 1:

Forward: 5'- CTG GCT CAA CTG CGG TAC AG -3'

Reverse: 5'- ACC AAT TCT GCA CAT CAC ATT CA -3

We isolated total RNA as previously described using the Trizol method. 300 ng of total RNA was reverse transcribed with the SuperScript First-Strand Synthesis System according to the manufacturer's instructions. (Invitrogen, San Diego, CA). Real-Time PCR was performed using the SYBR Green kit (Invitrogen) according to the manufacturer's instructions. Fluorescence was measured by the ABI 7700 thermocycler

(Agilent, Palo Alto, CA) and we analyzed the results in Excel (Microsoft, Redmond, WA). Fold-change of these genes was calculated using the ddCt method [13].

Results

Of the 12,473 probe sets present on the array, we detected 6,642 at E10.5 (53%), 5,621 at E12.5 (45.1%), 5,325 at E15.5 (42.7%) and 5,008 at E17.5 (40.2%). Of these we selected 622 probe sets, corresponding to 599 genes, for further analysis based on the criteria given above, e.g. four-fold up- or down- regulation between paired time points. This allowed us to focus on those genes most highly regulated by development, and eliminated noise created by unregulated genes from interfering with clustering and functional analysis.

At E10.5, 188 probe sets were up-regulated relative to E12.5. Several functional categories were observed to be over-represented. Most notable were genes involved in angiogenesis and blood vessel development, morphogenesis and organogenesis, and genes involved in lipid metabolism and transport. 108 probe sets were up-regulated at E 12.5 relative to E10.5. The over-represented functional classes include hormones, ribosomal proteins and genes involved in pregnancy.

When comparing E12.5 to E15.5, 208 probe sets were up-regulated at E12.5. The over-represented gene categories were involved in cell cycle control and RNA binding proteins. At E15.5, 86 probe sets were up-regulated when compared to E12.5. Genes involved in cellular transport were notably over-represented.

We also compared E15.5 to E17.5 and identified 40 probe sets up-regulated at E15.5. Most over-represented genes were involved in cellular transport and cell growth and

maintenance. At E17.5 61 probe sets were up-regulated. We noted an over-abundance of genes involved in the regulation of transcription and numerous proteins that localize to the nucleus.

Discussion

The molecular basis of placental development remains poorly understood. Recent studies have begun to shed some light on this process, and numerous genes have been demonstrated to be essential for the proper differentiation of placental cell lineages and fetal survival. Unfortunately, the details of the interactions and effects of these genes are unclear. The present study has identified several subsets of genes that are regulated during placental development. These genes can be grouped according to their expression patterns, which suggest that particular subsets of diverse genes are induced or repressed in concert at crucial times during placental ontogeny.

Microarray analysis has provided insights into the genetic mechanisms of cancer growth, development, responses to stress and, numerous other processes. Expression pattern analysis and gene clustering have provided insights into the functional classes of genes involved in these diverse processes,[14-16]. Our data set provides some insights into the regulation of placental development. We performed pairwise analysis between days E10.5 and E12.5, E12.5 and E15.5, E15.5 and E17.5. This has allowed us to follow the induction and repression of several gene classes at different stages of placental development.

Genes up-regulated early in placental development testify to the rapid tissue growth and cell proliferation occurring during this developmental period and the vascular

development that occurs. Genes involved in several key processes are strongly up-regulated at E10.5. These range from angiogenesis, to lipid metabolism, to cell cycle regulation. Genes such as ELK3, c-fos induced growth factor, plasminogen (the precursor to angiostatin), serine (or cysteine) proteinase inhibitor clade F member 1 are all involved in blood vessel morphogenesis and were up-regulated at E10.5. Cyclin D1, cyclin E2, cyclin C, MAD 2, pleiotrophin, Brca 2, which are involved in cell cycle control are also up-regulated strongly at E10.5 and E12.5. Ribosomal genes were notably up-regulated at E12.5. Several genes involved in lipid transport and metabolism were also detected: apolipoprotein B, apolipoprotein C-II, lysophospholipase, microsomal triglyceride transfer protein and adiponectin receptor 1. Lipid transport and metabolism are important for the proper development of the fetus [17] and disruption of lipid transporters leads to embryonic lethality [18, 19].

Previous null mutant experiments have identified several of these genes as embryonic lethal, further confirming their importance in placental development. For instance, Cops2 mutants died soon after implantation [20], Pten mutants died at E9.5 secondary to placental failure [8], and connexin 43 mutants died shortly after birth due to cardiac and vascular abnormalities [21].

Genes such as growth hormone releasing hormone prolactin-like protein I, secretin, and chorionic somatomammotropin hormone 2 were regulated by placental development. Recent studies in the sheep suggest that growth hormone releasing hormone is involved in the regulation of placental growth hormone and lactogen expression [22]. Insulin-like growth factor II and Insulin-like growth factor binding protein 2 were up-regulated from E10.5 to E12.5. These genes have been previously

shown to be expressed in the placenta [23]. In our study IGF-II is up-regulated at E12.5 and later. Previous studies have shown that after E12.5 it is mainly produced by trophoblast glycogen cells [24]. IGF-II also appears to have key functions in placental transport and permeability [25]. A number of prolactin-like proteins were shown to be regulated with development: prolactin-like protein C 1, prolactin-like protein F, prolactin-like protein I, prolactin like protein K. The prolactin gene family in the mouse has 26 identified members to date [26] and, several studies have shown that this gene family performs key reproductive and regulatory functions in the placenta [27-29].

The present study underscores the extreme complexity of placental development. Although our results provide useful leads for further investigation, they are far from complete. Several limitations include the use of the entire placenta which may mask processes occurring in subsets of placental cells. For example, our data disclosed no metabolic pathways to be regulated significantly. This could be due to the inherent noise generated by micro-arrays. Furthermore, the abundance of expressed sequence tags and genes of uncertain function renders thorough analysis difficult. The present study thus provides a starting point from which placental development can be studied in greater detail. Of particular interest, are those situations in which normal development is perturbed either by the mutation of a critical gene, or by the introduction of an external stressor such as nutrient restriction or hypoxia. Study of these stresses will allow the study of gene expression patterns in comparison to normal development. Such studies also would provide further insights into the structural and molecular adaptation to external stressors in the developing murine placenta and, hopefully, reveal more specific roles for some of the genes identified by the present study.

Perspective/Conclusions

Since their development, cDNA and oligo microarrays have proven to be powerful tools in the elucidation of gene expression patterns. Early work in yeast demonstrated the ability to examine cellular processes at the global gene expression level [30].

Several microarray studies have focused on the placenta and embryo. In the mouse, genes that were placenta specific were identified by comparing expression levels between the embryo and placenta at E12.5 [11]. Two human studies have described the *in-vitro* differentiation of trophoblast [10] and decidual cells [31]. These reports identified subsets of genes classified in five broad functional classes: cell and tissue structure, cell and tissue function, regulation of gene expression, expressed sequence tag, and "function unknown" that were up- and down-regulated during the process of differentiation.

As with most other organ systems, placental development remains an incompletely understood process. Placental failure accounts for numerous instances of fetal mortality [3], and may play a role in the genesis of preclampsia and intra-uterine growth restriction [32]. Numerous genes have been shown to be essential for proper placental function as an organ of respiratory gas and nutrient exchange and hormone synthesis. Results of the present study underscore the complexity of placental development, and emphasizes that numerous genes are crucial for successful placentation. The multiplicity of roles the placenta plays makes it difficult to dissect out the numerous regulatory pathways controlling these functions. Thus, a challenge for the future is to understand the genetic mechanisms underlying development of specific cell types as well as of the placenta as a whole.

Acknowledgement: We thank Brenda Kreutzer for her assistance in the preparation of this manuscript, and J.D. Heck of the DNA Array Core, University of California, Irvine, Irvine, CA for technical assistance.

REFERENCES

1. Simmons, D.G. and J.C. Cross. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. Dev Biol, 2005; 284: 12-24.

2. Cross, J.C. How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. Placenta, 2005; 26 Suppl A: S3-9.

3. Cross, J.C., D. Baczyk, N. Dobric, M. Hemberger, M. Hughes, D.G. Simmons, H. Yamamoto, and J.C. Kingdom. Genes, development and evolution of the placenta. Placenta, 2003; 24: 123-30.

4. Adams, R.H., A. Porras, G. Alonso, M. Jones, K. Vintersten, S. Panelli, A. Valladares, L. Perez, R. Klein, and A.R. Nebreda. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell, 2000; 6: 109-16.

5. Barak, Y., M.C. Nelson, E.S. Ong, Y.Z. Jones, P. Ruiz-Lozano, K.R. Chien, A. Koder, and R.M. Evans. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell, 1999; 4: 585-95.

6. Schreiber, J., E. Riethmacher-Sonnenberg, D. Riethmacher, E.E. Tuerk, J. Enderich, M.R. Bosl, and M. Wegner. Placental failure in mice lacking the mammalian homolog of glial cells missing, GCMa. Mol Cell Biol, 2000; 20: 2466-74.

7. Schorpp-Kistner, M., Z.Q. Wang, P. Angel, and E.F. Wagner. JunB is essential for mammalian placentation. Embo J, 1999; 18: 934-48.

8. Yamamoto, H., M.L. Flannery, S. Kupriyanov, J. Pearce, S.R. McKercher, G.W. Henkel, R.A. Maki, Z. Werb, and R.G. Oshima. Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev, 1998; 12: 1315-26.

9. Rossant, J. and J.C. Cross. Placental development: lessons from mouse mutants. Nat Rev Genet, 2001; 2: 538-48.

10. Aronow, B.J., B.D. Richardson, and S. Handwerger. Microarray analysis of trophoblast differentiation: gene expression reprogramming in key gene function categories. Physiol Genomics, 2001; 6: 105-16.

11. Tanaka, T.S., S.A. Jaradat, M.K. Lim, G.J. Kargul, X. Wang, M.J. Grahovac, S. Pantano, Y. Sano, Y. Piao, R. Nagaraja, H. Doi, W.H. Wood, 3rd, K.G. Becker, and M.S. Ko. Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. Proc Natl Acad Sci U S A, 2000; 97: 9127-32.

12. Downs, K.M. and T. Davies. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development, 1993; 118: 1255-66.

13. Livak, K.J. and T.D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001; 25: 402-8.

14. Chu, S., J. DeRisi, M. Eisen, J. Mulholland, D. Botstein, P.O. Brown, and I. Herskowitz. The transcriptional program of sporulation in budding yeast. Science, 1998; 282: 699-705.

15. Gasa, R., C. Mrejen, N. Leachman, M. Otten, M. Barnes, J. Wang, S. Chakrabarti, R. Mirmira, and M. German. Proendocrine genes coordinate the pancreatic islet differentiation program in vitro. Proc Natl Acad Sci U S A, 2004; 101: 13245-50.

16. Iyer, V.R., M.B. Eisen, D.T. Ross, G. Schuler, T. Moore, J.C. Lee, J.M. Trent, L.M. Staudt, J. Hudson, Jr., M.S. Boguski, D. Lashkari, D. Shalon, D. Botstein, and P.O. Brown. The transcriptional program in the response of human fibroblasts to serum. Science, 1999; 283: 83-7.

17. Shekhawat, P., M.J. Bennett, Y. Sadovsky, D.M. Nelson, D. Rakheja, and A.W. Strauss. Human placenta metabolizes fatty acids: implications for fetal fatty acid oxidation disorders and maternal liver diseases. Am J Physiol Endocrinol Metab, 2003; 284: E1098-105.

18. Gimeno, R.E., D.J. Hirsch, S. Punreddy, Y. Sun, A.M. Ortegon, H. Wu, T. Daniels, A. Stricker-Krongrad, H.F. Lodish, and A. Stahl. Targeted deletion of fatty acid transport protein-4 results in early embryonic lethality. J Biol Chem, 2003; 278: 49512-6.

19. Farese, R.V., Jr., S. Cases, S.L. Ruland, H.J. Kayden, J.S. Wong, S.G. Young, and R.L. Hamilton. A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal-fetal lipid transport in mice. J Lipid Res, 1996; 37: 347-60.

20. Lykke-Andersen, K., L. Schaefer, S. Menon, X.W. Deng, J.B. Miller, and N. Wei. Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death. Mol Cell Biol, 2003; 23: 6790-7.

21. Reaume, A.G., P.A. de Sousa, S. Kulkarni, B.L. Langille, D. Zhu, T.C. Davies, S.C. Juneja, G.M. Kidder, and J. Rossant. Cardiac malformation in neonatal mice lacking connexin43. Science, 1995; 267: 1831-4.

22. Lacroix, M.C., P. Bolifraud, D. Durieux, A. Pauloin, M. Vidaud, and G. Kann. Placental growth hormone and lactogen production by perifused ovine placental explants: regulation by growth hormone-releasing hormone and glucose. Biol Reprod, 2002; 66: 555-61.

23. Zollers, W.G., Jr., J.S. Babischkin, G.J. Pepe, and E.D. Albrecht. Developmental regulation of placental insulin-like growth factor (IGF)-II and IGF-binding protein-1 and -2 messenger RNA expression during primate pregnancy. Biol Reprod, 2001; 65: 1208-14.

24. Redline, R.W., C.L. Chernicky, H.Q. Tan, J. Ilan, and J. Ilan. Differential expression of insulin-like growth factor-II in specific regions of the late (post day 9.5) murine placenta. Mol Reprod Dev, 1993; 36: 121-9.

25. Sibley, C.P., P.M. Coan, A.C. Ferguson-Smith, W. Dean, J. Hughes, P. Smith, W. Reik, G.J. Burton, A.L. Fowden, and M. Constancia. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. Proc Natl Acad Sci U S A, 2004; 101: 8204-8.

26. Wiemers, D.O., L.J. Shao, R. Ain, G. Dai, and M.J. Soares. The mouse prolactin gene family locus. Endocrinology, 2003; 144: 313-25.

27. Ain, R., G. Dai, J.H. Dunmore, A.R. Godwin, and M.J. Soares. A prolactin family paralog regulates reproductive adaptations to a physiological stressor. Proc Natl Acad Sci U S A, 2004; 101: 16543-8.

28. Linzer, D.I. and S.J. Fisher. The placenta and the prolactin family of hormones: regulation of the physiology of pregnancy. Mol Endocrinol, 1999; 13: 837-40.

29. Ain, R., J.S. Tash, and M.J. Soares. Prolactin-like protein-A is a functional modulator of natural killer cells at the maternal-fetal interface. Mol Cell Endocrinol, 2003; 204: 65-74.

30. Spellman, P.T., G. Sherlock, M.Q. Zhang, V.R. Iyer, K. Anders, M.B. Eisen, P.O. Brown, D. Botstein, and B. Futcher. Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol Biol Cell, 1998; 9: 3273-97.

31. Brar, A.K., S. Handwerger, C.A. Kessler, and B.J. Aronow. Gene induction and categorical reprogramming during in vitro human endometrial fibroblast decidualization. Physiol Genomics, 2001; 7: 135-48.

32. Kingdom, J., B. Huppertz, G. Seaward, and P. Kaufmann. Development of the placental villous tree and its consequences for fetal growth. Eur J Obstet Gynecol Reprod Biol, 2000; 92: 35-43.

Table 1: Overexpressed Gene Ontology categories during placental development

Up at E10.5 vs E12.5		
Gene Name	**Genbank #**	**Function**
Cadherin 2	M31131	Blood Vessel Development
procollagen, type XVIII, alpha 1	AV112006	Blood Vessel Development
Elk3	L19953	Blood Vessel Development
c-fos induced growth factor	X99572	Blood Vessel Development
paired-like homeodomain transcription factor 2	U70132	Blood Vessel Development
plasminogen	AV028204	Blood Vessel Development
smoothened homolog (Drosophila)	AF089721	Blood Vessel Development
apolipoprotein A-IV	M64248	Lipid Transport
apolipoprotein C-II	Z22216	Lipid Transport
microsomal triglyceride transfer protein	L47970	Lipid Transport
apolipoprotein B	AI787317	Lipid Transport
apolipoprotein M	AA655303	Lipid Transport
oxysterol binding protein-like 1A	AB017026	Lipid Transport
Up at E12.5 vs E10.5		
growth hormone releasing hormone	M31658	Hormone
IGF-II	X71922	Hormone
chorionic somatomammotropin hormone 2	M14647	Hormone
prolactin like protein k	AI324118	Hormone
prolactin-like protein C 1	AF090140	Hormone
prolactin-like protein F	AF020524	Hormone
secretin	X73580	Hormone
proliferin 2	K03235	Hormone
ribosomal protein L26	X80699	Ribosomal Protein
ribosomal protein L27a	X05021	Ribosomal Protein
ribosomal protein L39	AI648758	Ribosomal Protein
ribosomal protein L41	U93862	Ribosomal Protein
ribosomal protein L7	M29015	Ribosomal Protein
ribosomal protein S23	AI837403	Ribosomal Protein
ribosomal protein, large, P1	U29402	Ribosomal Protein
ribosomal protein S16	M11408	Ribosomal Protein
ribosomal protein L23a	AI835315	Ribosomal Protein
Up at E12.5 vs 15.5		
cell division cycle 25 homolog C (S. cerevisiae)	L16926	Cell Cycle

cell division cycle 2 homolog A (S. pombe)	M38724	Cell Cycle
centrin 3	Y12474	Cell Cycle
chondroitin sulfate proteoglycan 6	Y15128	Cell Cycle
ect2 oncogene	L11316	Cell Cycle
SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	U42385	Cell Cycle
SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	AA032310	Cell Cycle
kinesin family member 11	AJ223293	Cell Cycle
annexin A1	AV003419	Cell Cycle
antigen identified by monoclonal antibody Ki 67	X82786	Cell Cycle
c-myc	L00039	Cell Cycle
phosphatase and tensin homolog	U92437	Cell Cycle
KH domain containing, RNA binding, signal transduction associated 1	U17961	Cell Cycle
stromal antigen 1	Z75332	Cell Cycle
stromal antigen 2	AJ002636	Cell Cycle
ubiquitin-activating enzyme E1C	AF077330	Cell Cycle
DnaJ (Hsp40) homolog, subfamily C, member 2	U53208	Cell Cycle
Septin 7	AJ223782	Cell Cycle
cyclin C	U62638	Cell Cycle
MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	U83902	Cell Cycle
exportin 1, CRM1 homolog (yeast)	AW123788	Nuclear Transport
fragile X mental retardation syndrome 1 homolog	L23971	Nuclear Transport
Heterogeneous nuclear ribonucleoprotein A1	AI183202	Nuclear Transport
karyopherin (importin) alpha 3	AW047023	Nuclear Transport
karyopherin (importin) beta 3	AI847564	Nuclear Transport
Sjogren syndrome antigen B	L00993	Nuclear Transport
replication factor C (activator 1) 4	AW122092	DNA metabolism
minichromosome maintenance deficient 4 homolog (S. cerevisiae)	D26089	DNA metabolism
ribonucleotide reductase M1	K02927	DNA metabolism
topoisomerase (DNA) II alpha	U01915	DNA metabolism
topoisomerase I	X70956	DNA metabolism
X-ray repair complementing defective repair in Chinese hamster cells 5	X66323	DNA metabolism
origin recognition complex, subunit 4-like (S. cerevisiae)	AI553463	DNA metabolism
single-stranded DNA binding protein	AA881160	DNA metabolism
suppressor of zeste 12 homolog (Drosophila)	AA692708	DNA metabolism
Smarca5	AA794509	DNA metabolism
Up at E15.5 vs E12.5		
quiescin Q6	AW123556	Transport
aminolevulinic acid synthase 2, erythroid	M15268	Transport
albumin 1	X13060	Transport

Mus musculus apolipoprotein A-I gene	U79573	Transport
apolipoprotein A-IV	M64248	Transport
apolipoprotein E	D00466	Transport
complement component 1, q subcomponent, alpha polypeptide	X58861	Transport
folate receptor 1	AV035020	Transport
nitric oxide synthase 2, inducible, macrophage	U43428	Transport
solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	X14309	Transport
solute carrier family 4 (anion exchanger), member 1	X02677	Transport
solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	AB017189	Transport
solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	AJ012754	Transport
solute carrier family 39 (zinc transporter), member 4	AI842734	Transport
transcobalamin 2	AF090686	Transport
tubulin, alpha 7	M13443	Transport
DNA segment, Chr 11, ERATO Doi 18, expressed	AI841330	Transport
carboxypeptidase X 1 (M14 family)	AF077738	Transport
syntaxin 6	AW124355	Transport
RIKEN cDNA 1300017J02 gene	AA895838	Transport
protein disulfide isomerase-related	AA755004	Transport
cytochrome P450, family 2, subfamily s, polypeptide 1	AW123273	Transport
Up at E17.5 vs E15.5		
calcyclin binding protein	U97327	Nuclear protein
expressed sequence AI314180	AW049625	Nuclear protein
hypothetical protein LOC234358	D10627	Nuclear protein
nucleolar protein 5	AF053232	Nuclear protein
RIKEN cDNA 4921531G14 gene	AW124329	Nuclear protein
RIKEN cDNA 1810007M14 gene	AI098965	Nuclear protein
nucleolar protein 7	AW124661	Nuclear protein
stromal antigen 1	Z75332	Nuclear protein (Cell cycle)
stromal antigen 2	AI846890	Nuclear protein (Cell cycle)
splicing factor, arginine/serine-rich 1 (ASF/SF2)	AI842878	Nuclear protein (RNA Transport)
fragile X mental retardation syndrome 1 homolog	L23971	Nuclear protein (RNA Transport)
heterogeneous nuclear ribonucleoprotein A1	AI846109	Nuclear protein (RNA Transport)
Sjogren syndrome antigen B	L00993	Nuclear protein (RNA Transport)
forkhead box A1	U44752	Nuclear protein (Transcription)
nuclear transcription factor-Y beta	X55316	Nuclear protein (Transcription)

SRY-box containing gene 11	AF009414	Nuclear protein (Transcription)
TATA box binding protein (Tbp)-associated factor, RNA polymerase I, B	Y09973	Nuclear protein (Transcription)
transcription elongation factor A (SII) 1	D00925	Nuclear protein (Transcription)
suppressor of zeste 12 homolog (Drosophila)	AA692708	Nuclear protein (Transcription)
transcription elongation factor B (SIII), polypeptide 1	AW045358	Nuclear protein (Transcription)
NMDA receptor-regulated gene 1	AW260482	Nuclear protein (Transcription)

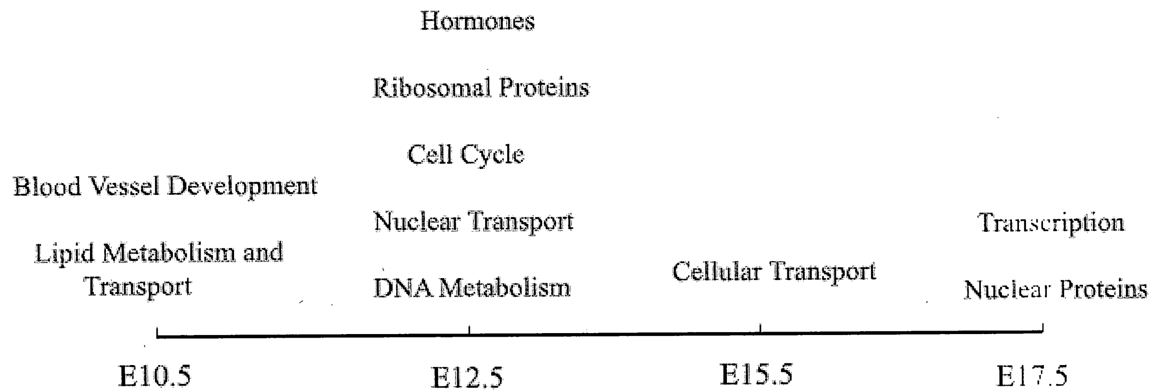

Figure 1: Over-represented gene functional classes throughout mouse placental development.

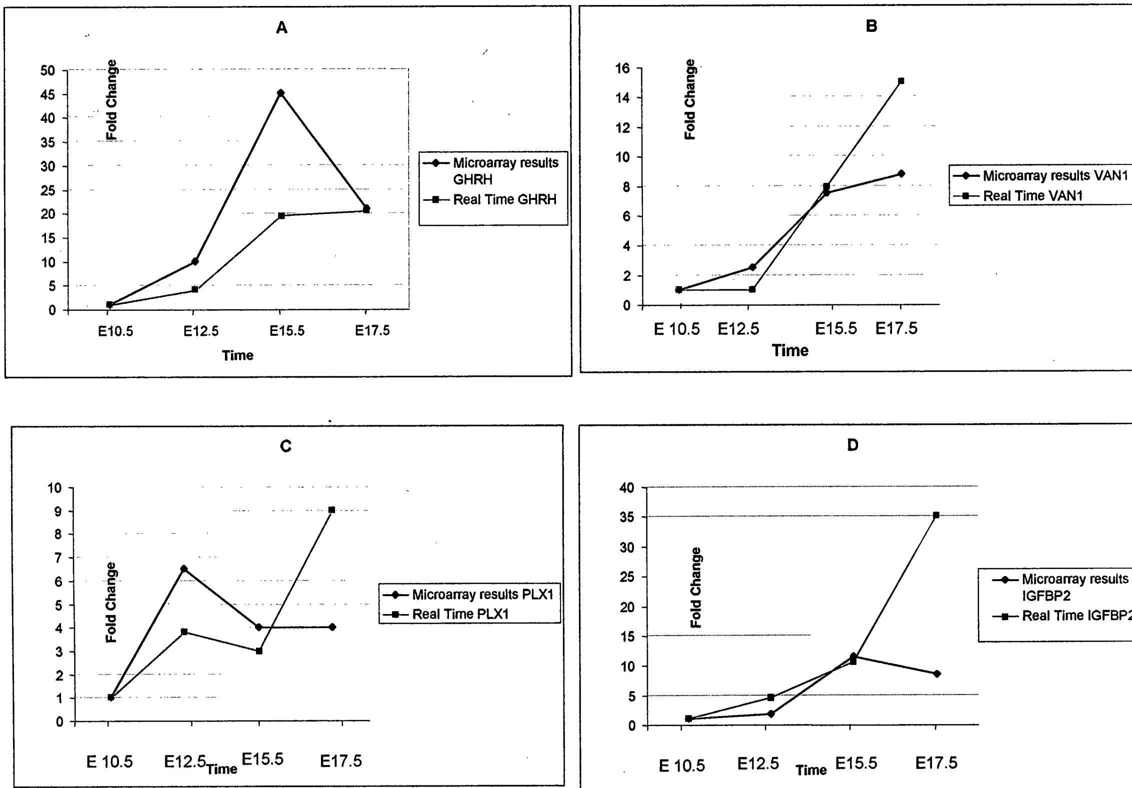

Figure 2: Comparison of the expression pattern of Growth Hormone Releasing Hormone (pannel A), Vanin 1 (pannel B), Placenta Specific Homeobox Gene 1 (pannel C) and, Insulin-Like Growth Factor binding protein 2 (pannel D), as determined by microarray and Real-Time PCR.

Chapter 3

Gene expression patterns in the hypoxic murine placenta: A possible role in epigenesis?

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Running Head: Hypoxia and placental gene expression

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Supported, in part by USPHS grant HD-03807 to LDL and the Society for Gynecological Investigation Medical Student Grant to CPG.

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Abstract

OBJECTIVE: Hypoxia has been identified as a major stressor in placental and fetal development. In order to comprehend more completely hypoxic stress responses we sought to measure gene expression changes in the mouse placenta in response to 48 hours of hypoxia.

METHODS: We exposed embryonic day 15.5 (E15.5) pregnant mice to 48 hours of hypoxia (10.5% O_2), following which we used the Affymetrix Mouse 430A_2.0 array to measure gene expression changes. We observed 171 probe sets, corresponding to 163 genes, that were regulated by hypoxia ($p < 0.01$). Ninety genes were up-regulated and 73 were down-regulated. We functionally annotated the regulated genes and examined over-represented functional categories.

RESULTS: Among the up- and down-regulated genes, we observed several over-represented functional categories. Up-regulated genes included those involved in metabolism, oxygen transport, proteolysis, cell death, metabolism of reactive oxygen species and DNA methylation. Genes involved in the regulation of transcription, cell cycle regulation and cell structure were down-regulated.

CONCLUSIONS: Microarray analysis has allowed us to describe the genetic response to hypoxia in the mouse placenta. The observation that hypoxia up-regulates reactive oxygen species metabolism in conjunction with DNA methylation enzymes suggests that hypoxia may contribute to long-term epigenetic changes in stressed tissues and organs.

KEY WORDS: Placenta, hypoxia, DNA methylation, proteolysis, granzymes.

Introduction

Successful placental development is crucial for the proper growth, maturation, and survival of the embryo/fetus. This is evidenced by the numerous lethal embryonic null-mutants secondary to placental failure [1-5]. Hypoxia has been identified as a major stressor in placental and fetal development, and is thought to be a contributing cause to placental pathology such as that in association with pre-eclampsia [6, 7], and can lead to low birth weight and intra-uterine growth restriction (IUGR) [8].

Little is known, however, about the adaptive mechanisms involved in the placental responses to hypoxia. Several recent studies have attempted to harness the power of microarray and proteomic analysis to elucidate responses to hypoxia in cultured human cytotrophoblasts [9], and in rat embryos and placentas [10]. As with other cells, oxygen regulates the normal development of placental trophoblast cells, which undergo differentiation and/or proliferation in response to varying oxygen concentrations. Acting through aryl receptor nuclear transporter and hypoxia inducible factor 1, oxygen regulates placental cell phenotypes and gene expression [11]. Hypoxic stress leads to placental cell death and dysregulation of vasculogenesis, which negatively affects the development of the placental vascular bed [12].

In response to long-term exposure to high altitude, the ovine placenta undergoes significant structural changes [13, 14], and its vasculature shows significant increases in capillary density, vessel tortuosity, and a decrease in diffusion distance from maternal to fetal blood [14]. The human placenta also shows significant morphologic and morphometric changes in response to high altitude, long-term hypoxia [15]. Nonetheless, little is known about the molecular basis of these changes. In the present study, we tested

the hypothesis that hypoxia-induced altered placental morphology is accompanied by significant changes in gene expression profiles

Materials and Methods

Animals.

Eight-week old FVB/NJ male and female mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed at the Animal Research Facility, Loma Linda University, Loma Linda, CA under conditions of 14 h light, 10 h darkness, ambient temperature of 20° C, and relative humidity of 30-60%. All experimental protocols were in compliance with the Animal Welfare Act, and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Breeding and tissue collection:

As previously described following breeding, the pregnant females were sacrificed by CO_2 asphyxiation at embryonic day 17.5 (E17.5). The uterus was removed rapidly and placed in a petri dish containing RNALater solution (Ambion, Austin, TX), placentae were isolated under a dissection microscope, and stored at 4°C in microcentrifuge tubes. We determined the developmental stages of the embryos by visual inspection according to a modified Theiler staging system [16]. Details of the staging system are available online at http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml. Placentae from each mouse were pooled, and total RNA isolated using the TRIZOL reagent kit (Life Technologies, Rockville, MD). The total RNA was stored at -80 °C in microfuge tubes.

Hypoxic Exposure

At E15 the pregnant mice were placed in a custom sealed plexyglass cage. A mixture of compressed air and nitrogen was pumped in and O_2 concentration was measured using the Oxychecq Expedition, an in-line oxygen meter (Ohychecq, Fort Pierce, FL). The mice were maintained in an environment of 10.5% O_2 (one-half atmosphere equivalent) for 48 hours. Oxygen concentration in the cages was checked at hourly intervals during the day, and every three hours at night to ensure a consistent hypoxic exposure.

Probe preparation, microarray hybridization, and data analysis:

The RNA was processed for use on the Affymetrix Mouse 430A_2.0 array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Briefly, 5 μg of total RNA was reverse transcribed to double stranded cDNA (Superscript II kit, Life Technologies). The double stranded cDNA was used in an in-vitro transcription reaction to generate biotynilated cRNA. The cRNA was purified, fragmented, and hybridized to the Affymetrix chip. Washes and staining were performed in an Affymetrix Gene Chip Fluidics station 400. The Affymetrix arrays were scanned using a Gene Array Scanner (Hewlett Packard, Austin, TX). and processed at the Microarray Facility, University of California Irvine, (Irvine, CA). Data analysis was performed using the GeneSpring software (Silicon Genetics, Santa Clara, CA), and hybridization intensities were normalized to the median value for each array. The hybridizations were performed in triplicate for control and hypoxic conditions. Analyses were performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam. We analysed the data using the random variance method at a significance of $p < 0.001$ [17].

The genes were assigned to functional classes based on the GO database (http://www.geneontology.org/GO.annotation.html) and significantly over-represented GO categories in the gene sets were analyzed using the Gene Ontology Tree Machine (http://genereg.ornl.gov/gotm/).

Real Time PCR

In an effort to validate the results of the microarray analysis, we randomly chose genes that were shown to be regulated by hypoxia for analysis using real time PCR. We retrieved intron spanning, verified real time PCR primers from Primer Bank (http://pga.mgh.harvard.edu/primerbank/index.html) and synthesized by Integrated DNA technologies (Coralville, CA). The primer sequences selected were:

Granzyme F :

Forward Primer GCTGGGGGAGAACATCCATC

Reverse Primer TGTCCTGTTTAGCCCATAGGT

Acetyl-Coenzyme A synthetase 2 :

Forward Primer GTTTGGGACACTCCTTACCATAC

Reverse Primer AGGCAGTTGACAGACACATTC

peroxiredoxin 2:

Forward Primer GGTAACGCGCAAATCGGAAAG

Reverse Primer TCCAGTGGGTAGAAAAAGAGGT

Cathepsin G:

Forward Primer AGGGTTTCTGGTGCGAGAAG

Reverse Primer GTTCTGCGGATTGTAATCAGGAT

Glial cells missing homolog 1:

Forward Primer AGAGATACTGAGCTGGGACATT

Reverse Primer CTGTCGTCCGAGCTGTAGATG

We isolated total RNA using the Trizol method. 300 ng of total RNA was reverse transcribed with the SuperScript First-Strand Synthesis System according to the manufacturer's instructions. (Invitrogen, San Diego, CA). Real-Time PCR was performed using the SYBR Green kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Fluorescence was measured by the The ABI 7700 thermocycler (Agilent), and we analyzed the results in Excel (Microsoft, Redmond, WA). Fold-change was calculated using the ddCt method [18].

Histochemistry

Placentae were harvested and fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated, embedded in paraffin, and cut into 5-μm sections. Adjacent sections were obtained for hematoxylin and eosin staining,

Results

As noted above, to evaluate the genetic response to hypoxic hypoxia we used the Affymetrix Mouse 430A_2.0 array to compare gene expression levels between normal placentas at E17.5, and placentas from mothers exposed to 48 hours of hypoxia. Of

22,690 genes present on the microarray, by use of the random variance model, we selected 209 probe sets, corresponding to 197 genes, that were regulated at a $p < 0.001$. One hundred and three were up-regulated by hypoxia, while 94 were down-regulated. Some of the most highly up-regulated genes were those related to metabolism (Alpha-keto reductase family-1 member 7, mitochondrial solute carrying protein, acetyl-coenzyme A synthetase 2, NADPH oxidase 4), oxygen transport (erythroid associated factor, hemoglobin Y beta-like embryonic chain, erythrocyte protein band 4.2), proteolysis (cathepsin G, kallikrein 4, dipeptidase 1, serine protease 32), cell death (Bcl-like 2, perforin 1, glutathione peroxidase 1), and metabolism of reactive oxygen species. We also noted that several genes related to DNA methylation and epigenetic control were up-regulated (DNA methyltransferase 3B, methyl-CpG binding domain protein 1, RNA binding motif protein 3) (Table 1 and Figure 1). Figure 2 shows the chromosomal distribution of genes up-regulated by hypoxia. We also noted an over-abundance of genes from chromosome 14 that were up-regulated as shown in figure 2. These correspond to the Granzyme family of proteases.

Among the down-regulated genes, most notable were several transcription factors (transformation related protein 63, doublesex and mab-3 related transcription factor 1, glial cells missing homolog 1, zinc finger, imprinted 1), cell cycle (cyclin M2), cell structure (keratin complex 2 basic, gene 8, procollagen C-proteinase enhancer protein, fibromodulin) (Table 2). Table 1 and 2 gives a complete list of these genes up- or down-regulated and their known function. We verified the regulation of several genes using real time PCR. These results are included in Table 3. As shown in Figure 3, we also noted that

placental morphology had changed in response to hypoxia. Qualitatively the hypoxic placentas appeared much more vascular and contained more red blood cells.

Discussion

Since their development, cDNA and oligo microarrays have proven to be powerful tools in the elucidation of gene expression patterns. Early work in the yeast demonstrated the ability to examine cellular processes at the global gene expression level [19, 20]. One study catalogued the hypoxic-induced response in the rat embryo to hypoxic exposure after both 24 hours and 11 days [10]. Several recent studies also have examined human trophoblast responses *in-vitro* to low oxygen tension [21, 22]. Also, gene expression changes in placentas from pregnancies complicated by pre-eclampsia and IUGR have been catalogued [23]. These studies have highlighted the diverse ways in which placental cells respond to hypoxic stress [21, 24].

In response to 48 hour exposure to hypoxia in the near-term mouse placenta, the present study has identified 90 genes that were up-regulated and 73 that were down-regulated. Several of these genes have been noted previously to be regulated by hypoxia and/or involved in angiogenesis and metabolic responses. For instance, NAPDH oxydase 4 has been identified as a potential O_2 sensor and regulator of the hypoxia-inducible factor HIF-1α[25]. Ferrocheloelastase is a protein involved in heme metabolism, and has been shown to be up-regulated by hypoxia [26]. Aminolevulinic acid synthase 2, glutathione peroxidase 1, and peroxiredoxin 2 are involved in the metabolism of reactive oxygen species, and their activity and expression levels, have previously been identified as regulated by hypoxia [27, 28]. Glycophorin A is involved in erythroid differentiation

and is regulated by erythropoietin [29]. Lactotransferrin is an antioxidant protein involved in iron metabolism and in scavenging of free radicals under hypoxic conditions [30].

Of note, several members of the granzyme gene family were up-regulated by hypoxia. Granzymes are serine proteases expressed by lymphocytes, and thought to be involved in T-cell-mediated cytotoxicity. Granzymes are released along with perforin by natural killer (NK) cells and cytotoxic T lymphocytes. They trigger apoptosis in target cells through several mechanisms. Several studies have indicated that granzymes are expressed in the placenta, and may play a broader role in placental development [31, 32]. Granzymes are organized in several gene clusters on mouse chromosomes 13 (granzyme A cluster), chromosome 14 (granzyme B cluster), and chromosome 10 (granzyme M cluster). In the present study, the granzymes up-regulated by hypoxia are all members of the granzyme B cluster on chromosome 14. This is reflected by the chromosomal distribution of the genes in our data set, which shows an over-abundance of chromosome 14 genes, when compared to the background frequency present on the Mouse 430A 2.0 array (Fig 2). This suggests either co-regulation at the promoter level or a potential epigenetic effect. A key question that requires further study is the extent to which these proteases play a role in the hypoxia-induced response. One possibility is that they mediate trophoblast apoptosis in response to hypoxic stress. Another explanation is that they contribute to extra-cellular matrix remodeling, which is an integral part of angiogenesis. In a previous study [33] we noted that granzyme B was up-regulated early in placental development. Currently, we show that hypoxia reactivates this gene later in development. We also have observed an up-regulation of perforin, which may indicate

that in response to hypoxia a greater number of NK cells invade the placenta. NK cells have been shown to mediate a number or important placental functions [34]. Thus, our data suggests that granzymes not only play a role in normal placental development, but also are involved in responses to hypoxia.

Also notable was the significant up-regulation of genes involved in DNA methylation and epigenetic control: DNA methyltransferase 3b and methyl CpG binding domain protein 1. Epigenetic modification is an important mechanism of gene expression regulation that does not involve sequence modification, but DNA and histone methylation. This results in changes in gene expression patterns that are of importance in development, genomic imprinting, and the development of cancer [35]. The generation of reactive oxygen species and the manipulation of glutathione metabolism has been shown to regulate a number of cellular processes including DNA methylation [36]. As depicted in Figure 4, this suggests a chain of events that commences with hypoxia, leads to elevated reactive oxygen species which, in turn, activates the DNA methylation machinery, and ultimately leads to long-term changes in the organism unrelated to nucleotide sequence modification. A key to unraveling this mechanism will be the identification of the targets for hypermethylation and subsequent long-term down-regulation of transcription.

The mouse placenta appears to respond to hypoxia through several adaptive mechanisms. These include up-regulation of genes associated with erythropoiesis, increases in heme and iron metabolism and, in genes involved in proteolysis and peptidolysis. These varied responses suggest that the placenta responds by increasing its oxygen carrying capacity, increasing metabolic and antioxidant responses, and initiating

tissue growth, turnover and remodeling. Studies in the human and the sheep have demonstrated that the placenta undergoes several morphological and genetic changes in response to prolonged hypoxia [13, 14, 21]. Hypoxic insults secondary to preeclampsia, maternal smoking, or exposure to high altitude can contribute to placental insufficiency and may lead to intrauterine growth restriction [37-40]. The exact mechanisms of these changes are not understood. Hopefully, the present study will provide insights at the molecular level on these important clinical problems. Extracellular matrix remodeling, the modulation of apoptosis, altered cellular metabolism, and epigenetic changes all appear to be crucial steps in the physiological adaptations of the mouse placenta to hypoxia.

Perspective/Conclusions

In the present study we examined the responses in the mouse placenta to 48 hours of hypoxia. The gene-regulated responses in the placenta to hypoxic exposure is an important process that may shed light on the progression of several pathologies linked to infant morbidity and death. Few studies have catalogued these responses, and our results provide an overview of the genes involved in this hypoxic-induced response. The placenta appears to respond through several adaptive mechanisms: increased tissue remodeling, erythrocyte production, increased metabolism, and mediation of apoptosis. Several studies have suggested that disturbances in the heme and iron metabolism may result in some of the pathologies involved in placental dysfunction [41]. Other work has highlighted disturbances in apoptotic cell turnover in pre-eclamptic pregnancies [42]. The

present study points to a family of proteins, previously described in the immune system, as potentially involved in the response of the mouse placenta to hypoxia.

Quite obviously, further studies will be needed to classify these hypoxic-induced responses in greater detail, and several key questions must be answered. For instance, what function do these proteins have in placental growth, differentiation, and metabolism? What upstream regulators trigger their up-regulation? In particular, which O_2 regulated transcription factors regulate the expression of these genes? In addition, gene responses at an earlier time points may shed light on the early regulators of this response.

The present study also emphasises the intriguing possibility that hypoxic stress may trigger epigenetic changes at the level of DNA methylation. If so, this is potentially very important. The present findings may provide a mechanism to explain the effect of "fetal programming" and the prenatal origin of adult disease, that has been shown to be of great importance to society.

Acknowledgement: We thank Brenda Kreutzer for her assistance in the preparation of this manuscript, and J.D. Heck of the DNA Array Core, University of California, Irvine, Irvine, CA for technical assistance.

Table 1 Genes up- regulated by hypoxia belonging to significantly over represented functional categories (genes significant to $p < 0.001$).

Affy ID	Gene Name	Fold Change	Functional Annotation
1430453_a_at	Bcl2-like 2	1.5	Cell Death
1449130_at	CD1d1 antigen	1.8	Cell Death
1422622_at	nitric oxide synthase 3, endothelial cell	1.6	Cell Death
1451862_a_at	perforin 1 (pore forming protein)	1.9	Cell Death
1450015_x_at	sphingosine-1-phosphate phosphatase 1	1.6	Cell Death
1417088_at	zinc finger protein 346	1.4	Cell Death
1419060_at	granzyme B	3.3	Cell Death/Proteolysis
1421256_at	granzyme C	2.7	Cell Death/Proteolysis
1420344_x_at	granzyme D	2.0	Cell Death/Proteolysis
1421227_at	granzyme E	2.2	Cell Death/Proteolysis
1418679_at	granzyme F	2.7	Cell Death/Proteolysis
1422867_at	granzyme G	2.4	Cell Death/Proteolysis
1418351_a_at	DNA methyltransferase 3B	1.7	DNA methylation
1453678_at	methyl-CpG binding domain protein 1	3.2	DNA methylation
1451675_a_at	aminolevulinic acid synthase 2, erythroid	1.8	Heme biosynthesis
1418698_a_at	ferrochelatase	2.2	Heme biosynthesis
1424798_a_at	a disintegrin and metalloprotease domain 5	2.0	Proteolysis
1419594_at	cathepsin G	4.2	Proteolysis
1450652_at	cathepsin K	1.6	Proteolysis
1420763_at	kallikrein 4 (prostase, enamel matrix, prostate)	2.3	Proteolysis
1423542_at	kallikrein 7 (chymotryptic, stratum corneum)	3.0	Proteolysis
1420844_at	ubiquilin 2	2.6	Proteolysis
1452954_at	ubiquitin-conjugating enzyme E2C	2.0	Proteolysis
1419161_a_at	NADPH oxidase 4	2.1	ROS metabolism
1415964_at	stearoyl-Coenzyme A desaturase 1	1.9	ROS metabolism
1450956_at	stearoyl-coenzyme A desaturase 3	1.8	ROS metabolism
1418506_a_at	peroxiredoxin 2	1.7	ROS metabolism
1460671_at	glutathione peroxidase 1	1.7	ROS metabolism/Cell Death

Table 2
Genes down-regulated by hypoxia*

Affy Id #	Gene Name	Fold Change	Gene Symbol
1451512_s_at	3-hydroxyisobutyryl-Coenzyme A hydrolase	1.5	Hibch
1426657_s_at	3-phosphoglycerate dehydrogenase	1.7	Phgdh
1449363_at	activating transcription factor 3	1.6	Atf3
1438651_a_at	angiotensin receptor-like 1	1.7	Agtrl1
1451731_at	ATP-binding cassette, sub-family A (ABC1), member 3	1.6	Abca3
1423572_at	Bcl2-like 2	1.5	Bcl2l2
1422609_at	cAMP-regulated phosphoprotein 19	1.7	Arpp19
1420008_s_at	cDNA sequence BC037006	1.8	BC037006
1435375_at	cDNA sequence BC052328	1.7	BC052328
1418983_at	channel-interacting PDZ domain protein	1.5	Cipp
1420249_s_at	chemokine (C-C motif) ligand 6	2.7	Ccl6
1419627_s_at	C-type lectin domain family 4, member n	2.0	Clec4n
1450756_s_at	cullin 3	1.6	Cul3
1423475_at	cyclin M2	2.5	Cnnm2
1435820_x_at	discoidin domain receptor family, member 1	1.9	Ddr1
1423065_at	DNA methyltransferase 3A	1.6	Dnmt3a
1416010_a_at	EH-domain containing 1	1.8	Ehd1
1449167_at	erythrocyte protein band 4.1-like 4a	1.5	Epb4.1l4a
1416514_a_at	fascin homolog 1, actin bundling protein (Strongylocentrotus) purpuratus)	2.1	Fscn1
1448747_at	F-box only protein 32	1.6	Fbxo32
1437718_x_at	fibromodulin	1.6	Fmod
1456084_x_at	fibromodulin	2.2	Fmod
1428280_at	FIP1 like 1 (S. cerevisiae)	1.5	Fip1l1
1435551_at	formin-family protein FHOS2	1.9	FHOS2
1418374_at	FXYD domain-containing ion transport regulator 3	1.9	Fxyd3
1424647_at	gamma-aminobutyric acid (GABA-A)	1.8	Gabrp

	receptor, pi		
1423554_at	gamma-glutamyl carboxylase	2.1	Ggcx
1438887_a_at	germ cell-less homolog (Drosophila)	2.1	Gcl
1420601_at	glial cells missing homolog 1 (Drosophila)	2.2	Gcm1
1453604_a_at	Hbs1-like (S. cerevisiae)	1.8	Hbs1l
1431274_a_at	heat shock protein, A	1.8	Hspa9a
1460017_at	hypothetical LOC434179	1.7	LOC434179
1417933_at	insulin-like growth factor binding protein 6	2.7	Igfbp6
1451798_at	interleukin 1 receptor antagonist	2.0	Il1rn
1429063_s_at	kinesin family member 16B	1.6	Kif16b
1415855_at	kit ligand	1.5	Kitl
1421459_a_at	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	2.4	Lrp8
1426696_at	low density lipoprotein receptor-related protein associated protein 1	1.6	Lrpap1
1423547_at	lysozyme	3.0	Lyzs
1438676_at	macrophage activation 2 like	2.1	Mpa2l
1422975_at	membrane metallo endopeptidase	1.9	Mme
1422580_at	myosin, light polypeptide 4	2.0	Myl4
1456028_x_at	Myristoylated alanine rich protein kinase C substrate	1.6	Marcks
1438625_s_at	necdin /// PCTAIRE-motif protein kinase 1	1.6	Ndn /// Pctk1
1423516_a_at	nidogen 2	2.0	Nid2
1419663_at	osteoglycin	2.3	Ogn
1421113_at	pepsinogen 5, group I	2.1	Pga5
1436970_a_at	platelet derived growth factor receptor, beta polypeptide	1.8	Pdgfrb
1448254_at	pleiotrophin	1.8	Ptn
1423856_at	popeye domain containing 3	2.4	Popdc3
1422293_a_at	potassium channel	1.7	Kctd1

	tetramerisation domain containing 1		
1437165_a_at	procollagen C-proteinase enhancer protein	2.1	Pcolce
1449529_s_at	prolactin-like protein E	2.5	Prlpe
1416321_s_at	proline arginine-rich end leucine-rich repeat	1.8	Prelp
1460419_a_at	protein kinase C, beta 1	2.1	Prkcb1
1426811_at	protein phosphatase 2, regulatory subunit B (B56), beta isoform	1.6	Ppp2r5b
1452127_a_at	protein tyrosine phosphatase, non-receptor type 13	1.6	Ptpn13
1424314_at	PRP3 pre-mRNA processing factor 3 homolog (yeast)	1.7	Prpf3
1434914_at	RAB6B, member RAS oncogene family	1.9	Rab6b
1419586_at	retinitis pigmentosa 2 homolog (human)	1.6	Rp2h
1417400_at	retinoic acid induced 14	1.6	Rai14
1415737_at	riboflavin kinase	1.5	Rfk
1428657_at	RIKEN cDNA 1110037N09 gene	1.5	1110037N09Rik
1424524_at	RIKEN cDNA 1200002N14 gene	1.7	1200002N14Rik
1449327_at	RIKEN cDNA 1600015I10 gene	3.1	1600015I10Rik
1453132_a_at	RIKEN cDNA 1810036H07 gene	1.7	1810036H07Rik
1423705_at	RIKEN cDNA 2310057D15 gene	2.1	2310057D15Rik
1456117_at	RIKEN cDNA 2600005C20 gene	1.9	2600005C20Rik
1428357_at	RIKEN cDNA 2610019F03 gene	1.5	2610019F03Rik
1427112_at	RIKEN cDNA 2700049H19 gene	1.7	2700049H19Rik
1451320_at	RIKEN cDNA 3110043J09 gene	1.6	3110043J09Rik
1427067_at	RIKEN cDNA 4933439F18 gene	1.7	4933439F18Rik
1416574_at	RIKEN cDNA 5730589K01 gene	1.5	5730589K01Rik
1455764_at	RIKEN cDNA B930007L02 gene	1.9	B930007L02Rik
1423540_at	RNA binding motif, single stranded interacting protein 2	1.6	Rbms2

1456733_x_at	serine (or cysteine) proteinase inhibitor, clade H, member 1	2.3	Serpinh1
1439200_x_at	Similar to erythroid differentiation regulator	2.8	
1453928_a_at	Sjogren syndrome antigen B	1.6	Ssb
1434235_at	solute carrier family 20, member 2	1.9	Slc20a2
1417639_at	solute carrier family 22 (organic cation transporter), member 4	1.9	Slc22a4
1426722_at	solute carrier family 38, member 2	1.6	Slc38a2
1420504_at	solute carrier family 6 (neurotransmitter transporter), member 14	2.2	Slc6a14
1426008_a_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	2.1	Slc7a2
1448793_a_at	syndecan 4	1.5	Sdc4
1426546_at	testis-specific kinase 2	1.6	Tesk2
1422967_a_at	transferrin receptor	3.6	Tfrc
1418158_at	transformation related protein 63	2.0	Trp63
1416009_at	transmembrane 4 superfamily member 8	1.7	Tm4sf8
1420923_at	ubiquitin specific protease 9, X chromosome	1.8	Usp9x
1420994_at	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	1.8	B3gnt5
1438975_x_at	zinc finger, DHHC domain containing 14	1.5	Zdhhc14
1421405_at	zinc finger, imprinted 1	2.4	Zim1
1424594_at		1.6	
1448847_at		1.8	

* All genes significant to $p < 0.001$

Table 3 Real time PCR confirmation of select gene transcripts.

Gene name	Genbank #	Microarray change	PCR change
Granzyme F	NM_010374	Up	Up
Acetyl-Coenzyme A synthetase 2	NM_080575	Up	Up
Peroxiredoxin 2	NM_011563	Up	Up
Cathepsin G	NM_007800	Up	Up
Glial cells missing homolog 1	NM_008103	Down	Down

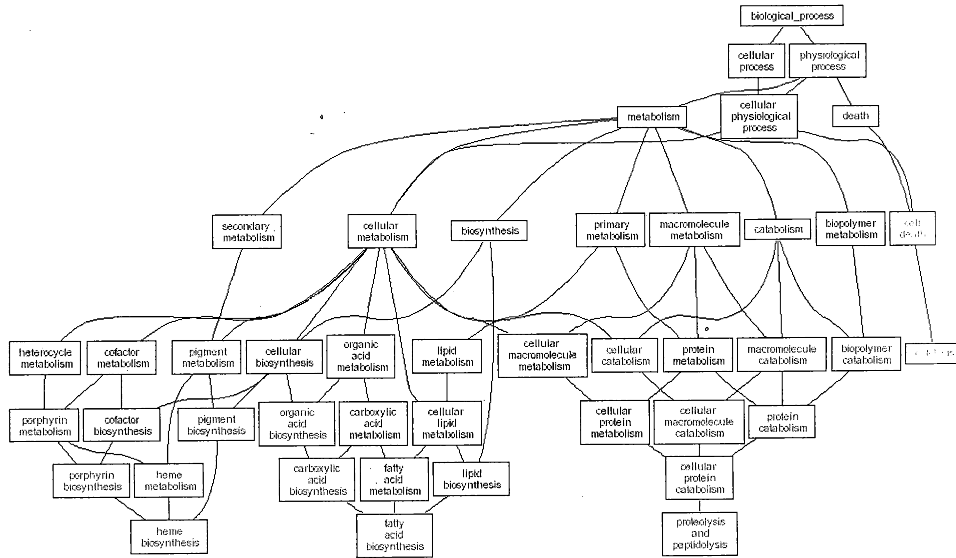

Figure 1. Over represented functional categories in the genes up-regulated by hypoxia.

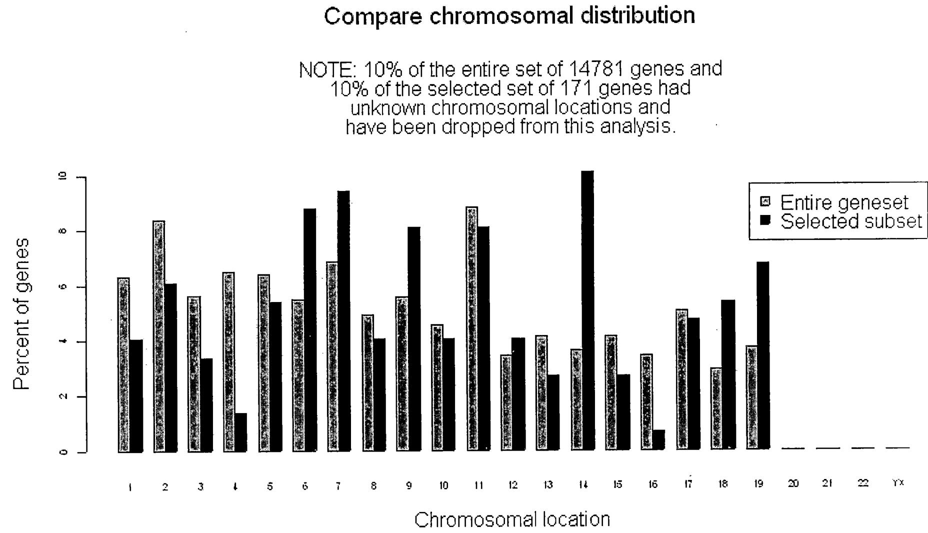

Figure 2. Chromosomal Distribution of the genes up-regulated by Hypoxia.

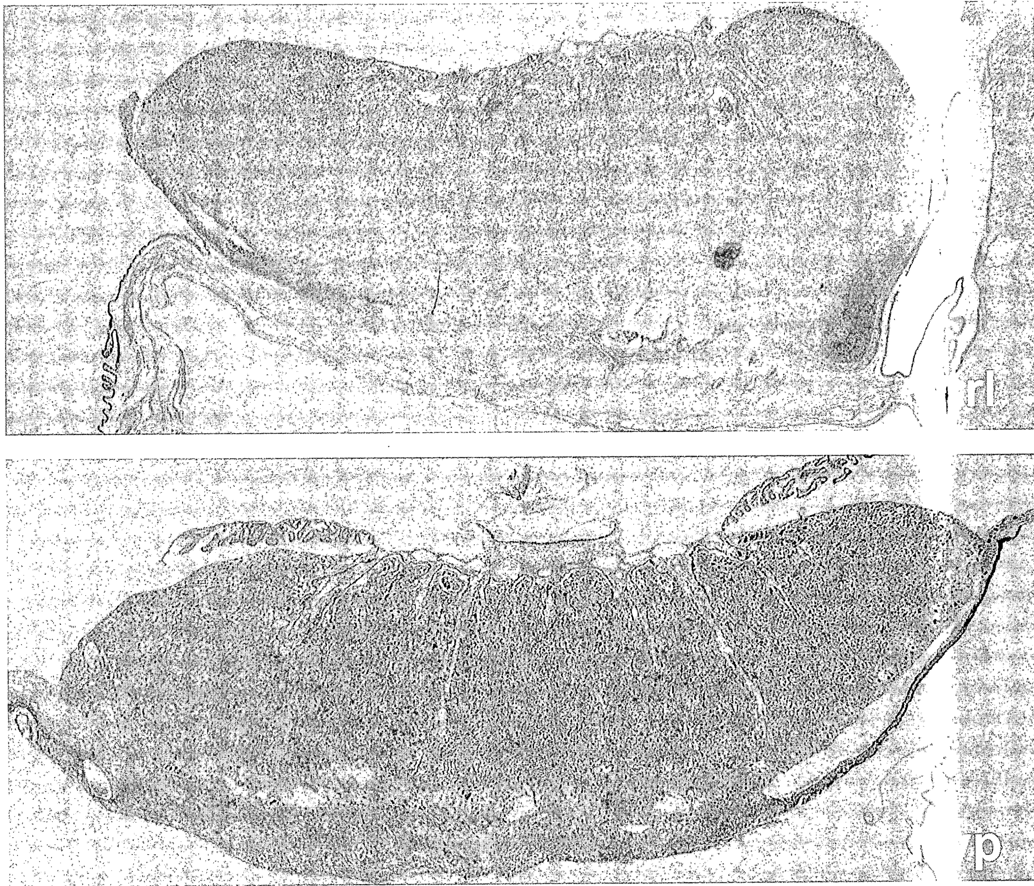

Figure 3. Histological changes in placental vasculature in response to hypoxia.

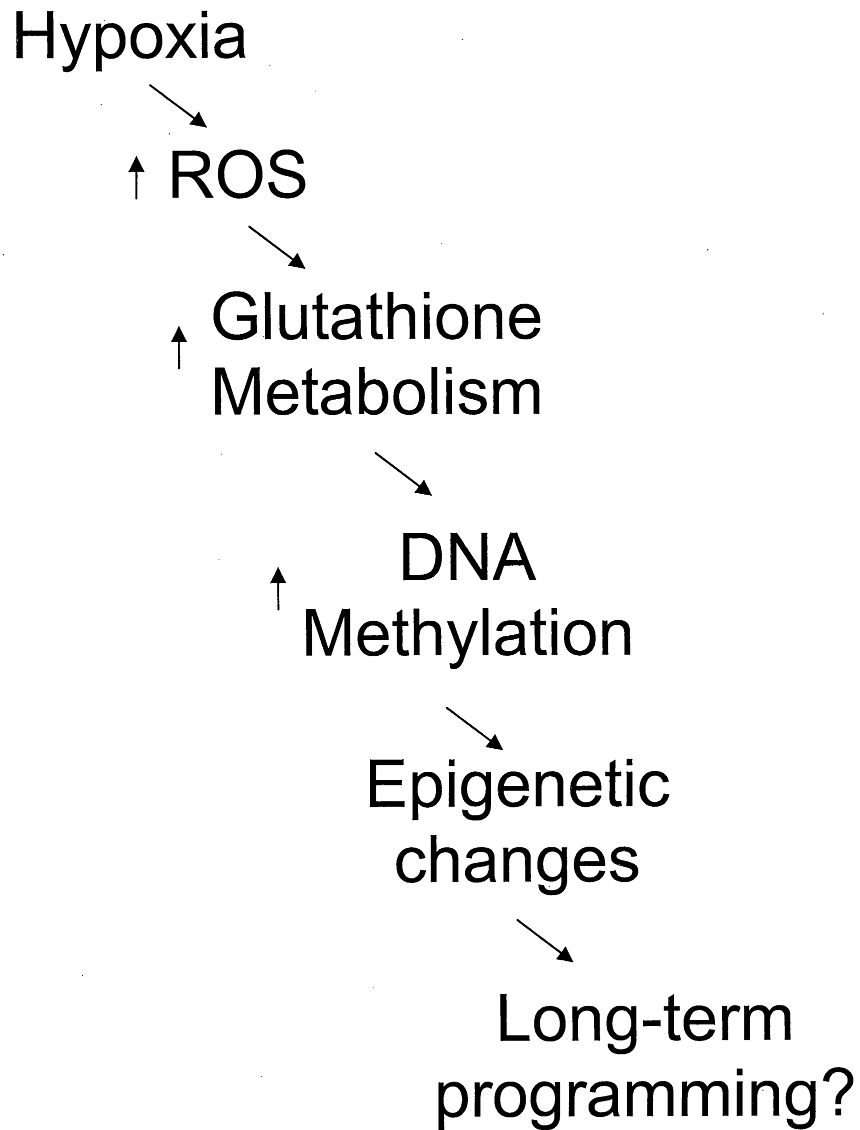

Figure 4. Proposed model for epigenetic changes in gene expression in response to hypoxia

References

1. Adams, R.H., A. Porras, G. Alonso, M. Jones, K. Vintersten, S. Panelli, A. Valladares, L. Perez, R. Klein, and A.R. Nebreda. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell, 2000; 6: 109-16.

2. Barak, Y., M.C. Nelson, E.S. Ong, Y.Z. Jones, P. Ruiz-Lozano, K.R. Chien, A. Koder, and R.M. Evans. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell, 1999; 4: 585-95.

3. Schreiber, J., E. Riethmacher-Sonnenberg, D. Riethmacher, E.E. Tuerk, J. Enderich, M.R. Bosl, and M. Wegner. Placental failure in mice lacking the mammalian homolog of glial cells missing, GCMa. Mol Cell Biol, 2000; 20: 2466-74.

4. Schorpp-Kistner, M., Z.Q. Wang, P. Angel, and E.F. Wagner. JunB is essential for mammalian placentation. Embo J, 1999; 18: 934-48.

5. Yamamoto, H., M.L. Flannery, S. Kupriyanov, J. Pearce, S.R. McKercher, G.W. Henkel, R.A. Maki, Z. Werb, and R.G. Oshima. Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev, 1998; 12: 1315-26.

6. Austgulen, R., C.V. Isaksen, L. Chedwick, P. Romundstad, L. Vatten, and C. Craven. Pre-eclampsia: associated with increased syncytial apoptosis when the infant is small-for-gestational-age. J Reprod Immunol, 2004; 61: 39-50.

7. Challier, J.C. and S. Uzan. [The human placenta and its pathologies: focus on oxygen]. Med Sci (Paris), 2003; 19: 1111-20.

8. Zamudio, S. The placenta at high altitude. High Alt Med Biol, 2003; 4: 171-91.

9. Hoang, V.M., R. Foulk, K. Clauser, A. Burlingame, B.W. Gibson, and S.J. Fisher. Functional proteomics: examining the effects of hypoxia on the cytotrophoblast protein repertoire. Biochemistry, 2001; 40: 4077-86.

10. Huang, S.T., K.C. Vo, D.J. Lyell, G.H. Faessen, S. Tulac, R. Tibshirani, A.J. Giaccia, and L.C. Giudice. Developmental response to hypoxia. Faseb J, 2004; 18: 1348-65.

11. Adelman, D.M., M. Gertsenstein, A. Nagy, M.C. Simon, and E. Maltepe. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev, 2000; 14: 3191-203.

12. Kingdom, J., B. Huppertz, G. Seaward, and P. Kaufmann. Development of the placental villous tree and its consequences for fetal growth. Eur J Obstet Gynecol Reprod Biol, 2000; 92: 35-43.

13. Penninga, L. and L.D. Longo. Ovine placentome morphology: effect of high altitude, long-term hypoxia. Placenta, 1998; 19: 187-93.

14. Krebs, C., L.D. Longo, and R. Leiser. Term ovine placental vasculature: comparison of sea level and high altitude conditions by corrosion cast and histomorphometry. Placenta, 1997; 18: 43-51.

15. Zhang, E.G., G.J. Burton, S.K. Smith, and D.S. Charnock-Jones. Placental vessel adaptation during gestation and to high altitude: changes in diameter and perivascular cell coverage. Placenta, 2002; 23: 751-62.

16. Downs, K.M. and T. Davies. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development, 1993; 118: 1255-66.

17. Wright, G.W. and R.M. Simon. A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics, 2003; 19: 2448-55.

18. Livak, K.J. and T.D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001; 25: 402-8.

19. Spellman, P.T., G. Sherlock, M.Q. Zhang, V.R. Iyer, K. Anders, M.B. Eisen, P.O. Brown, D. Botstein, and B. Futcher. Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol Biol Cell, 1998; 9: 3273-97.

20. Eisen, M.B., P.T. Spellman, P.O. Brown, and D. Botstein. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A, 1998; 95: 14863-8.

21. Roh, C.R., V. Budhraja, H.S. Kim, D.M. Nelson, and Y. Sadovsky. Microarray-based identification of differentially expressed genes in hypoxic term human trophoblasts and in placental villi of pregnancies with growth restricted fetuses. Placenta, 2005; 26: 319-28.

22. Nelson, D.M., S.D. Smith, T.C. Furesz, Y. Sadovsky, V. Ganapathy, C.A. Parvin, and C.H. Smith. Hypoxia reduces expression and function of system A amino acid transporters in cultured term human trophoblasts. Am J Physiol Cell Physiol, 2003; 284: C310-5.

23. Soleymanlou, N., I. Jurisica, O. Nevo, F. Ietta, X. Zhang, S. Zamudio, M. Post, and I. Caniggia. Molecular evidence of placental hypoxia in preeclampsia. J Clin Endocrinol Metab, 2005; 90: 4299-308.

24. Chen, C.P., K.G. Wang, C.Y. Chen, C. Yu, H.C. Chuang, and H. Chen. Altered placental syncytin and its receptor ASCT2 expression in placental development and pre-eclampsia. Bjog, 2006; 113: 152-8.

25. Zhu, H., T. Jackson, and H.F. Bunn. Detecting and responding to hypoxia. Nephrol Dial Transplant, 2002; 17 Suppl 1: 3-7.

26. Liu, Y.L., S.O. Ang, D.A. Weigent, J.T. Prchal, and J.R. Bloomer. Regulation of ferrochelatase gene expression by hypoxia. Life Sci, 2004; 75: 2035-43.

27. Abu-Farha, M., J. Niles, and W.G. Willmore. Erythroid-specific 5-aminolevulinate synthase protein is stabilized by low oxygen and proteasomal inhibition. Biochem Cell Biol, 2005; 83: 620-30.

28. Mysore, T.B., T.A. Shinkel, J. Collins, E.J. Salvaris, N. Fisicaro, L.J. Murray-Segal, L.E. Johnson, D.A. Lepore, S.N. Walters, R. Stokes, A.P. Chandra, P.J. O'Connell, A.J. d'Apice, and P.J. Cowan. Overexpression of glutathione peroxidase with two isoforms of superoxide dismutase protects mouse islets from oxidative injury and improves islet graft function. Diabetes, 2005; 54: 2109-16.

29. Gubin, A.N., J.M. Njoroge, G.G. Bouffard, and J.L. Miller. Gene expression in proliferating human erythroid cells. Genomics, 1999; 59: 168-77.

30. Morris, C.J., J.R. Earl, C.W. Trenam, and D.R. Blake. Reactive oxygen species and iron--a dangerous partnership in inflammation. Int J Biochem Cell Biol, 1995; 27: 109-22.

31. Hirst, C.E., M.S. Buzza, V.R. Sutton, J.A. Trapani, K.L. Loveland, and P.I. Bird. Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction. Mol Hum Reprod, 2001; 7: 1133-42.

32. Allen, M.P. and M. Nilsen-Hamilton. Granzymes D, E, F, and G are regulated through pregnancy and by IL-2 and IL-15 in granulated metrial gland cells. J Immunol, 1998; 161: 2772-9.

33. Gheorghe, C., S. Mohan, and L.D. Longo. Gene expression patterns in the developing murine placenta. J Soc Gynecol Investig, 2006; 13: 256-62.

34. Parham, P. NK cells and trophoblasts: partners in pregnancy. J Exp Med, 2004; 200: 951-5.

35. Turek-Plewa, J. and P.P. Jagodzinski. The role of mammalian DNA methyltransferases in the regulation of gene expression. Cell Mol Biol Lett, 2005; 10: 631-47.

36. Fratelli, M., L.O. Goodwin, U.A. Orom, S. Lombardi, R. Tonelli, M. Mengozzi, and P. Ghezzi. Gene expression profiling reveals a signaling role of glutathione in redox regulation. Proc Natl Acad Sci U S A, 2005; 102: 13998-4003.

37. Reshetnikova, O.S., G.J. Burton, and A.P. Milovanov. Effects of hypobaric hypoxia on the fetoplacental unit: the morphometric diffusing capacity of the villous membrane at high altitude. Am J Obstet Gynecol, 1994; 171: 1560-5.

38. Spira, A., E. Philippe, N. Spira, J. Dreyfus, and D. Schwartz. Smoking during pregnancy and placental pathology. Biomedicine, 1977; 27: 266-70.

39. Levy, R. and D.M. Nelson. To be, or not to be, that is the question. Apoptosis in human trophoblast. Placenta, 2000; 21: 1-13.

40. Jones, C.J. and H. Fox. An ultrastructural and ultrahistochemical study of the human placenta in maternal pre-eclampsia. Placenta, 1980; 1: 61-76.

41. Barber, A., S.C. Robson, L. Myatt, J.N. Bulmer, and F. Lyall. Heme oxygenase expression in human placenta and placental bed: reduced expression of placenta endothelial HO-2 in preeclampsia and fetal growth restriction. Faseb J, 2001; 15: 1158-68.

42. Huppertz, B., J. Kingdom, I. Caniggia, G. Desoye, S. Black, H. Korr, and P. Kaufmann. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta, 2003; 24: 181-90.

Chapter 4

Maternal protein restriction in the pregnant mouse: and gene expression in the placenta

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Running Head: Genetic response to dietary protein restriction

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Abstract

OBJECTIVE: Maternal protein restriction has been shown to have deleterious effects on placental development and has long term consequences for the progeny. In order to comprehend more completely stress responses to maternal protein restriction, we sought to measure gene expression changes in the mouse placenta in response to 7 days of protein restriction.

METHODS: We exposed pregnant mice from embryonic day 10.5 (E10.5) to E17.5, to a isocaloric diet containing 50% less protein than normal chow, following which we used the Affymetrix Mouse 430A_2.0 array to measure gene expression changes. We observed 244 probe sets, corresponding to 235 genes, that were regulated by protein restriction ($p < 0.01$). Ninety-one genes were up-regulated and 153 were down-regulated. We functionally annotated the regulated genes and examined over-represented functional categories.

RESULTS: Among the up- and down-regulated genes, we observed several over-represented functional categories. Up-regulated genes included those involved in apoptosis, negative regulators of cell growth, negative regulators of cell metabolism and genes related to epigenetic control. Genes involved in nucleotide metabolism were down-regulated.

CONCLUSIONS: Microarray analysis has allowed us to describe the genetic response to maternal protein deprivation in the mouse placenta. We observed that negative regulators of cell growth and metabolism in conjunction with genes involved in epigenesis were up-regulated, suggesting that protein deprivation may contribute to growth restriction and long-term epigenetic changes in stressed tissues and organs.

Introduction

During pregnancy nutritional deprivation of the mother may have deleterious consequences for the progeny. Historical data point to these effects in human populations. During WWII, the people of both Holland and Russia were subjected to severe dietary restrictions due to interdiction of food supplies by the German army [1, 2]. The children born under these conditions were not only small for gestational age, but they also developed health problems later in life [3-5]. Several major sequelae have been described including those of the cardiovascular system, type II diabetes, and mood and personality disorders [6].

Nutritional deprivation influences placental growth and morphology, alters the hormonal milieu of the developing fetus, and causes subsequent cardiovascular, hormonal and behavioral consequences in the adult [7, 8]. These epidemiologic observations have led to speculation regarding the mechanism of changes in the placenta, and their effects on the developing fetus. The observations made in human subjects have been confirmed in animal models. An important question is the extent to which these observed effects result from an overall caloric restriction, as opposed to a qualitative component in the diet that triggers the responses. Evidence from animal models points to protein deprivation as a major factor in these defects. For example, in the rat the growth reducing effects of a low calorie diet can only be reversed by a dietary increase in protein levels; vitamin supplements and caloric increases through carbohydrates did not reverse the effects observed [9]. Other studies have reveled that dietary amino acid balance is a key

mediator of some of the cardiovascular and metabolic effects observed in response to protein deprivation.

These studies indicate that nutritional deprivation, and protein restriction in particular, can have immediate deleterious effects on the fetus and the placenta, and may result in long-term effects that extend into adulthood. In the present study we hypothesized that, through the use of microarray technology, we could identify cellular pathways in the developing placenta that are responsive to protein deprivation, and propose a potential mechanism for the observed morphological and physiological changes.

Materials and Methods

Animals.

Eight-week old FVB/NJ male and female mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed at the Animal Research Facility, Loma Linda University, Loma Linda, CA under conditions of 14 h light, 10 h darkness, ambient temperature of 20° C, and relative humidity of 30-60%. All experimental protocols were in compliance with the Animal Welfare Act, and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Breeding and tissue collection:

The mice were bred by overnight monogamous pairing of virgin females with a male. The male was removed in the morning and that day was considered embryonic day 0.5

(E0.5). At E17.5 the pregnant females were sacrificed by CO_2 asphyxiation. The uterus was removed rapidly and placed in a petri dish containing RNALater solution (Ambion, Austin, TX). The placentae were isolated under a dissection microscope, and stored at 4°C in microcentrifuge tubes. We determined the developmental stages of the embryos by visual inspection according to a modified Theiler staging system [10]. Details of the staging system are available online at http://genex.hgu.mrc.ac.uk/Databases/Anatomy/MAstaging.shtml. Placentae from each mouse were pooled, and total RNA isolated using the TRIZOL reagent kit (Life Technologies, Rockville, MD). The total RNA was stored at -80 °C.

Protein Restriction:

The mice were initially fed a normal mouse chow (20% protein content by weight). At E10.5 the pregnant mice were switched to a custom protein diet (10% protein by weight) (Teklad, Indianapolis, IA). The diets were designed to ensure that mice would receive the same amount of calories and nutrients, but a reduced amount of protein. Maternal food intake and maternal weights were measured in order to assure isocaloric food intake.

Probe preparation, microarray hybridization, and data analysis:

The RNA was processed for use on the Affymetrix Mouse 430A_2.0 array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Briefly, 5 μg of total RNA was reverse transcribed to double stranded cDNA (Superscript II kit, Life Technologies). The double stranded cDNA was used in an in-vitro transcription reaction

to generate biotynilated cRNA. The cRNA was purified, fragmented, and hybridized to the Affymetrix chip. Washes and staining were performed in an Affymetrix Gene Chip Fluidics station 400. The Affymetrix arrays were scanned using a Gene Array Scanner (Hewlett Packard, Austin, TX). and processed at the Microarray Facility, University of California Irvine, (Irvine, CA). Data analysis was performed using the GeneSpring software (Silicon Genetics, Santa Clara, CA), and hybridization intensities were normalized to the median value for each array. The hybridizations were performed in triplicate for control and hypoxic conditions. Analyses were performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam. We analysed the data using the random variance method at a significance of $p < 0.001$ [11].

The genes were assigned to functional classes based on the GO database (http://www.geneontology.org/GO.annotation.html) and significantly over-represented GO categories in the gene sets were analyzed using the Gene Ontology Tree Machine (http://genereg.ornl.gov/gotm/).

Real Time PCR

In an effort to validate the results of the microarray analysis, we randomly chose several genes that were shown to be regulated by protein restriction for analysis using real time PCR. We retrieved verified real time PCR primers from Primer Bank (http://pga.mgh.harvard.edu/primerbank/index.html). The primers were synthesized by Integrated DNA technologies (Coralville, CA). The primer sequences selected were:

p53

Forward Primer CTCTCCCCCGCAAAAGAAAAA

Reverse Primer CGGAACATCTCGAAGCGTTTA

Cebpa

Forward Primer GCGTCCCAAACAGGTGACA

Reverse Primer CTCCAGGGAGAAGCCATTCTT

Hipk2

Forward Primer GAGACGGGAACCAAGTCAAAG

Reverse Primer GGGATTGGCTAAGGAAAGAGTG

Histone deacetylase 7a

Forward Primer GAACTCTTGAGCCCTTGGACA

Reverse Primer GGTGTGCTGCTACTACTGGG

Toll like receptor 3

Forward Primer AGCCAGAACTGTGCCAAATAC

Reverse Primer CCGTTCCCAACTTTGTAGATGA

Cyclophilin F

Forward Primer CTTCCACAGGGTGATCCCAG

Reverse Primer ACTGAGAGCCATTGGTGTTGG

Results

As noted above, to evaluate the genetic response to protein deprivation we used the Affymetrix Mouse 430A_2.0 oligonucleotide array to compare gene expression levels between normal placentas at E17.5 and those from pregnancies in which the mothers were exposed to seven days of protein deprivation. Of 22,690 genes present on the microarray, using the random variance model (Wright, 2003), we selected 244 probe sets, corresponding to 235 genes, that were regulated at a $p < 0.001$. As a consequence of maternal protein deprivation 91 of these probe sets were up-regulated, while 153 were down-regulated. As noted in Table 1, among the gene ontology classes most over-represented in the up-regulated group, we noted regulators of apoptosis (Bcl2-like 2, p53, endophilin, Fas-activated serine/threonine kinase), negative regulators of cell growth (farnesyltransferase CAAX box beta, cadherin 5, CCAAT/enhancer binding protein (C/EBP) alpha, inositol polyphosphate-5-phosphatase D, p53), and negative regulators of cellular metabolism (nuclear receptor co-repressor 2, histone deacetylase 7A, SPEN homolog, transcriptional regulator). We also noted that a number of genes involved in the p53 oncogene pathway and the mitogen-activated protein kinase pathway were up-regulated. Among down-regulated genes, particularily striking were those genes related to nucleotide metabolism (see Table 2).

Discussion

Several "models" or "experiments" in humans provide useful lessons on the effects of caloric restriction/malnutrition on fetal development and disease prevalence in

adulthood. The first is that of the "Hunger Winter" in Amsterdam and Western Holland from November 1944 until the Allied victory in May 1945. During this seven month period, the caloric ration fell from over 2000 to 400-800 calories per day, less than 25% of the recommended intake for adults. Although children, and to some extent pregnant and lactating women, received extra rations during the early part of this disastrous famine, they too suffered severe malnutrition [12]. In essence, as adults the infants that were small at birth had significantly greater prevalence of cardiovascular disease and diabetes [12-14]. Those fetuses exposed to maternal caloric restriction in mid-gestation had a much greater incidence of bronchitis and other pulmonary disease [15] and renal disease as evidenced by microalbuminuria [16]. Females conceived during the famine also had a much higher prevalence of obesity as adults [3]. The mechanisms of these in utero "programming" effects are unknown.

An additional rich source of epidemiologic data derives from the studies of David J. P. Barker and colleagues [7, 17-19]. A series of epidemiologic studies in several countries have correlated maternal dietary deficiencies that result in the newborn infant being small for gestational age or growth restricted with the prevalence of cardiovascular disease, diabetes, and numerous other conditions in the adult. Because various tissues and organ systems undergo critical, often brief, periods of growth and development during fetal life [20], such "programming" should not be unexpected with insults to the developing organism (whether be the stress of nutrient deprivation, hypoxia, or other) to have consequences later in life's course.

Studies in ruminants also have demonstrated that under-nutrition can have profound consequences for the fetus. In sheep, restricted maternal nutrition in early to

mid-gestation was associated with an increase in placental weight, an increase in crown-rump length, and lower fetal to placental weight ratios [21]. Maternal under-nutrition also caused an alteration of cardiovascular homeostatic regulation by the renin-angiotensin system, and exposed the lambs to higher levels of glucocorticoids [22]. These hormonal effects also were associated with hypertension in the lambs [23]. Protein restriction in bovines also caused an increase in placental weight and an altered placental morphology [24].

Studies in rodents have shown similar effects. Maternal protein restriction in rats triggers hypertension in the pups in adulthood [25]. These effects appear to be mediated through a suppression of the renin-angiotensin system in the pups [26]. An alteration of placental glucocorticoid metabolism also was observed in placentae of rats fed a protein restricted diet. The activity of 11β-hydoxysteroid dehydrogenase, an enzyme present in the placenta, which normally protects the pups from maternal glucocorticoid excess, was reduced in protein restricted rats [27]. Another hormonal alteration in nutritionally deprived rats was an increase in somatostatin expression in the periventricular nucleus of the pups. This led to much lower levels of growth hormone and had deleterious effects on the growth of the pups post-partum [28]. Fetal undernourishment also led to neuronal sequelae. The facial motor nucleus in pups was under-developed, and led to a functional decrease in the ability of pups to suckle and chew [29].

In a previous study, we used the Affymetrix oligonucleotide array to define changes in gene expression from E10.5 to E17.5 in the mouse placenta [30]. In addition, we have reported on significant changes in mouse placental gene expression in response

to maternal hypoxia for 48 hours, from E15.5 to E17.5 (see Chapter 3, paper submitted for publication).

In the present study of gene regulation in the murine placenta in response to maternal dietary protein restriction, we demonstrate a profound down-regulation of cell growth and proliferation and an up-regulation of genes coding for apoptotic proteins (Tables 1 and 2). Of particular interest, p53 along with Cebpa and Hipk2 (two proteins in the p53 pathway, an important regulator of cell growth and proliferation) were up-regulated. This pathway serves as a G1 checkpoint, and arrests growth and/or induces apoptosis in response to cellular damage. Mutations in the p53 gene have been implicated in a number of cancers and other pathological processes [31]. Hipk2, an upstream regulator of p53, activates its transcriptional activity and pro-apoptotic activities through phosporylation at Ser 46 [32]. Cebpa, is a transcription factor induced by p53, and mediates some of the downstream effects of p53 activation [33].

A potentially important finding of the present study, is that protein deprivation altered the expression of several genes involved in DNA methylation, histone acetylation, and epigenetic regulation of gene expression. The expression levels of histone deacetylase 7A and methionine adenosyltransferase II, alpha were significantly elevated. Histone actetylation triggers changes in chromatin structure, and regulates transcriptional availability of genes. In turn, histone deacetylation increases histone affinity for DNA, thereby repressing transcription [34]. Methionine adenosyltransferase II, alpha synthesizes AdoMet the direct precursor used for DNA methylation by methyltransferases [35]. Histone 2 (h3c2) is down-regulated, along with Mcm6 and

telomeric repeat binding factor 1. These proteins contribute to DNA replication, stability, and structure [36, 37].

In several animal models, in addition to potential deleterious effects, a positive aspect of nutritional deprivation in the adult is that of prolonged lifespan and reduced cancer rates. A proposed mechanism for these benefits is that nutritional restriction (without malnutrition) inhibits cellular proliferation and induces apoptosis. This effect has been shown in mice lacking p53, in which -/- and +/- mutants have lowered spontaneous cancer rates when fed a complete, but calorically reduced diet [38]. In the adult and aging animal, nutritional restriction has been shown to have beneficial effects that increased life span [39]. However, a different picture has emerged in the fetus. As discussed above, caloric and protein deprivation have been shown to trigger fetal programming of adult disease, and lead to an increased prevalence of metabolic disorders in adulthood [19, 40, 41]. In the developing fetus, numerous animal studies have shown the negative long-term effects caloric and protein deprivation on the cardiovascular, renal, nervous system and metabolism (for review see [42]). Both fetal and placental growth are essential for the long-term well-being of the individual. Thus, one would anticipate that profound inhibition of cellular growth at key time points during development would have grave long-term consequences for the embryo/fetus. This suggests that the timing of the treatment is a key determinant in the effect on the organism.

Perspective/Conclusions

Based on epidemiological evidence, Barker and his colleagues proposed the hypothesis of the fetal origin of adult disease [7]. Briefly stated, this hypothesis postulates that stress during key periods of fetal development "programs" the fetus for disease in adulthood. Placental insufficiency [43], hypoxia [44, 45], nutrient restriction or a low protein diet [46] have all been shown to impair development of the fetus, and possibly trigger genetic, functional and structural modifications that can lead to predisposition to disease in the adult.

Maternal starvation and amino acid deprivation have been shown to inhibit hepatic growth, trigger cell cycle arrest in G1, and reduce DNA synthesis [47]. In the rat kidney, studies have shown that fetal growth restriction triggers an increase in apoptosis, and alters p53 promoter methylation and histone acetylation [48, 49]. These changes are believed to trigger long-term changes in kidney function , and potentially to account for renal and cardiovascular disease later in life.

Overall, our data support the idea that maternal protein restriction, triggers an up-regulation of apoptosis-related genes, an increase in the p53 pathway, a change in epigenetic modulators, and an overall down-regulation of cellular proliferation and growth. The sheer abundance of inhibitory transcription factors, and other key negative cellular regulators, that are affected by protein deprivation offers a picture of profound and global down-regulation of the entire cellular proliferative machinery. These results suggest numerous avenues for future research, and raise a number of fundamental questions regarding energy/protein balance, cellular growth, and the cellular mechanisms of epigenesis.

Acknowledgement: We thank Brenda Kreutzer for her assistance in the preparation of this manuscript. This work was supported, in part by USPHS grant HD-03807 to LDL and the SGI Medical Student Grant to CPG.

Table 1

Genes Up-regulated by protein deprivation

1451895_a_at	24-dehydrocholesterol reductase	Dhcr24
1423556_at	aldo-keto reductase family 1, member B7	Akr1b7
1423526_at	AT rich interactive domain 3B (Bright like)	Arid3b
1425227_a_at	ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	Atp6v0a1
1430453_a_at	Bcl2-like 2	Bcl2l2
1422047_at	cadherin 5	Cdh5
1451896_a_at	calcium homeostasis endoplasmic reticulum protein	Cherp
1422450_at	catenin src	Catns
1418982_at	CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa
1451359_at	cDNA sequence BC005662	BC005662
1424726_at	cDNA sequence BC014685	BC014685
1419833_s_at	centaurin, delta 3	Centd3
1425204_s_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19a /// DEAD (Asp-Glu-Ala-Asp) box polypeptide 19b	Ddx19a /// Ddx19b
1421143_at	diaphanous homolog 1 (Drosophila)	Diap1
1422037_at	distal-less homeobox 3	Dlx3
1450475_at	distal-less homeobox 3	Dlx3
1419502_at	DNA segment, Chr 11, Lothar Hennighausen 1, expressed	D11Lgp1e
1421032_a_at	DnaJ (Hsp40) homolog, subfamily B, member 12	Dnajb12
1452100_at	Dullard homolog (Xenopus laevis)	Dullard
1452273_at	expressed sequence AA409316	AA409316
1434309_at	farnesyltransferase, CAAX box, beta	Fntb
1460635_at	Fas-activated serine/threonine kinase	Fastk
1449849_a_at	F-box and leucine-rich repeat protein 6	Fbxl6
1418396_at	G-protein signalling modulator 3 (AGS3-like, C. elegans)	Gpsm3
1417637_a_at	high mobility group 20 B	Hmg20b
1420813_at	histone deacetylase 7A	Hdac7a
1422799_at	HLA-B associated transcript 2	Bat2
1429566_a_at	homeodomain interacting protein kinase 2	Hipk2
1424195_a_at	inositol polyphosphate-5-phosphatase D	Inpp5d
1418265_s_at	interferon regulatory factor 2	Irf2
1448668_a_at	interleukin-1 receptor-associated kinase 1	Irak1
1452327_at	IQ motif and Sec7 domain 1	Iqsec1
1426873_s_at	junction plakoglobin	Jup
1420639_at	junction-mediating and regulatory protein	MGI:1913096
1428881_at	kinesin 2	Kns2
1426648_at	MAP kinase-activated protein kinase 2	Mapkapk2
1437226_x_at	MARCKS-like protein	Mlp
1456702_x_at	methionine adenosyltransferase II, alpha	Mat2a
1417472_at	myosin, heavy polypeptide 9, non-muscle	Myh9
1452254_at	myotubularin related protein 9	Mtmr9
1428367_at	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	Ndst1
1448893_at	nuclear receptor co-repressor 2	Ncor2
1424952_at	OCIA domain containing 1	Ociad1

1427550_at	paternally expressed 10	Peg10
1449851_at	period homolog 1 (Drosophila)	Per1
1418078_at	proteaseome (prosome, macropain) 28 subunit, 3	Psme3
1418341_at	RAB4A, member RAS oncogene family	Rab4a
1450903_at	RAD23b homolog (S. cerevisiae)	Rad23b
1425266_a_at	RAP1, GTP-GDP dissociation stimulator 1	Rap1gds1
1455984_at	retinoic acid induced 17	Rai17
1438507_x_at	ribosomal protein L14	Rpl14
1416033_at	RIKEN cDNA 1110006I15 gene	1110006I15Rik
1423816_at	RIKEN cDNA 1110012O05 gene	1110012O05Rik
1431213_a_at	RIKEN cDNA 1300007C21 gene /// similar to Retrovirus-related POL polyprotein (Endonuclease)	1300007C21Rik /// LOC433762
1460609_at	RIKEN cDNA 2810013E07 gene	2810013E07Rik
1431752_a_at	RIKEN cDNA 2900073H19 gene	2900073H19Rik
1431753_x_at	RIKEN cDNA 2900073H19 gene	2900073H19Rik
1427979_at	RIKEN cDNA 4732418C07 gene	4732418C07Rik
1437180_at	RIKEN cDNA 6530403A03 gene	6530403A03Rik
1448251_at	RIKEN cDNA 9030425E11 gene	9030425E11Rik
1424956_at	RIKEN cDNA D030015G18 gene	D030015G18Rik
1434438_at	SAM domain and HD domain, 1	Samhd1
1416294_at	secretory carrier membrane protein 3	Scamp3
1422167_at	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	Sema5a
1419329_at	SH3 domain protein 4	Sh3d4
1418011_a_at	SH3-domain GRB2-like B1 (endophilin)	Sh3glb1
1449005_at	solute carrier family 16 (monocarboxylic acid transporters), member 3	Slc16a3
1452139_at	solute carrier family 35, member C1	Slc35c1
1418326_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	Slc7a5
1420397_a_at	SPEN homolog, transcriptional regulator (Drosophila)	Spen
1425787_a_at	synaptotagmin-like 3	Sytl3
1423616_at	TAR (HIV) RNA binding protein 2	Tarbp2
1451586_at	testis enhanced gene transcript	Tegt
1452768_at	testis expressed gene 261	Tex261
1419954_s_at	testis expressed gene 27	Tex27
1421148_a_at	Tial1 cytotoxic granule-associated RNA binding protein-like 1	Tial1
1460743_at	tigger transposable element derived 5	Tigd5
1438501_at	Transcribed locus, moderately similar to XP_526987.1 similar to actin related protein 2/3 complex, subunit 5-like [Pan troglodytes]	
1426538_a_at	transformation related protein 53	Trp53
1448412_a_at	TSC22 domain family 4	Tsc22d4
1434930_at	two pore channel 1	Tpcn1

1451771_at	two pore channel 1	Tpcn1
1448370_at	Unc-51 like kinase 1 (C. elegans)	Ulk1
1438165_x_at	vesicle amine transport protein 1 homolog (T californica)	Vat1
1423726_at	vesicle amine transport protein 1 homolog (T californica)	Vat1
1420834_at	vesicle-associated membrane protein 2	Vamp2
1448121_at	WW domain binding protein 2	Wbp2
1438938_x_at		
1456618_at		
1450918_s_at		
1418670_s_at		

Table 2

Genes down-regulated by protein deprivation

1422716_a_at	acid phosphatase 1, soluble	Acp1
1438170_x_at	adhesion regulating molecule 1	Adrm1
1423781_at	amyloid beta precursor protein binding protein 1	Appbp1
1449459_s_at	ankyrin repeat and SOCS box-containing protein 13	Asb13
1416065_a_at	ankyrin repeat domain 10	Ankrd10
1439456_x_at	ATPase, H+ transporting, lysosomal accessory protein 2	Atp6ap2
1437688_x_at	ATPase, H+ transporting, lysosomal accessory protein 2	Atp6ap2
1450732_a_at	bicaudal D homolog 2 (Drosophila)	Bicd2
AFFX-BioB-M_at	biotin synthesis, sulfur insertion?	bioB
1436885_a_at	calcium homeostasis endoplasmic reticulum protein	Cherp
1456576_x_at	CCR4-NOT transcription complex, subunit 2	Cnot2
1451232_at	CD151 antigen	Cd151
1424093_x_at	CD151 antigen	Cd151
1456085_x_at	CD151 antigen	Cd151
1437670_x_at	CD151 antigen	Cd151
1420010_at	CDNA clone MGC:102317 IMAGE:6466398, complete cds	
1425646_at	cDNA sequence BC016495	BC016495
1419403_at	cDNA sequence BC017612	BC017612
1451518_at	cDNA sequence BC021921	BC021921
1435240_at	cDNA sequence BC053917	BC053917
1423682_a_at	cell division cycle associated 4	Cdca4
1436838_x_at	coactosin-like 1 (Dictyostelium)	Cotl1
1456175_a_at	coatomer protein complex, subunit beta 2 (beta prime)	Copb2
1454781_x_at	COMM domain containing 9	Commd9
1437982_x_at	COX15 homolog, cytochrome c oxidase assembly protein (yeast)	Cox15
1434705_at	C-terminal binding protein 2	Ctbp2
1454149_a_at	cyclin L2	Ccnl2
1437773_x_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	Ddx17
1438371_x_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Ddx5
1454793_x_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Ddx5
1439012_a_at	deoxycytidine kinase	Dck
AFFX-r2-Ec-bioD-3_at	dethiobiotin synthetase	bioD
1419915_at	DNA segment, Chr 10, ERATO Doi 438, expressed	D10Ertd438e
1449339_at	DNA segment, Chr 10, ERATO Doi 641, expressed	D10Ertd641e
1421050_at	DNA segment, Chr 11, Wayne State University 68, expressed	D11Wsu68e
1416236_a_at	epithelial V-like antigen 1	Eva1
1460652_at	Estrogen related receptor, alpha	Esrra
1424013_at	eukaryotic translation termination factor 1	Etf1
1439411_a_at	exportin 7	Xpo7

1455809_x_at	expressed sequence AI114950	AI114950
1455912_x_at	Expressed sequence AW538196	AW538196
1434108_at	F-box protein 11	Fbxo11
1452247_at	fragile X mental retardation gene 1, autosomal homolog	Fxr1h
1439150_x_at	GH regulated TBC protein 1	Grtp1
1419072_at	glutathione S-transferase, mu 7	Gstm7
1416855_at	growth arrest specific 1	Gas1
1418106_at	hairy/enhancer-of-split related with YRPW motif 2	Hey2
1434047_x_at	heterogeneous nuclear ribonucleoprotein A2/B1	Hnrpa2b1
1437099_x_at	Heterogeneous nuclear ribonucleoprotein F	Hnrpf
1422155_at	histone 2, H3c2	Hist2h3c2
1455777_x_at	hydroxysteroid (17-beta) dehydrogenase 4	Hsd17b4
1423033_at	intergral membrane protein 1	Itm1
1424746_at	Kinesin family member 1C	Kif1c
1449099_at	LPS-responsive beige-like anchor	Lrba
1416343_a_at	lysosomal membrane glycoprotein 2	Lamp2
1455978_a_at	matrilin 2	Matn2
1434378_a_at	Max dimerization protein 4	Mxd4
1422498_at	melanoma antigen, family H, 1	Mageh1
1436390_a_at	Mid-1-related chloride channel 1	Mclc
1438852_x_at	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	Mcm6
1455787_x_at	multiple inositol polyphosphate histidine phosphatase 1	Minpp1
1456381_x_at	myeloid cell leukemia sequence 1	Mcl1
1450543_at	myosin 1H	Myo1h
1456028_x_at	Myristoylated alanine rich protein kinase C substrate	Marcks
1438955_x_at	peptidylprolyl isomerase F (cyclophilin F)	Ppif
1455860_at	phosphatidylinositol glycan, class H	Pigh
1420132_s_at	Pituitary tumor-transforming 1 interacting protein	Pttg1ip
1425721_at	pleckstrin homology domain interacting protein	Phip
1417319_at	poliovirus receptor-related 3	Pvrl3
1451740_at	polyadenylate binding protein-interacting protein 1	Paip1
1456270_s_at	preferentially expressed antigen in melanoma like 6	Pramel6
1437845_x_at	Protein O-fucosyltransferase 2	Pofut2
1425537_at	protein phosphatase 1A, magnesium dependent, alpha isoform	Ppm1a
1451225_at	protein tyrosine phosphatase, non-receptor type 11	Ptpn11
1455105_at	protein tyrosine phosphatase, non-receptor type 12	Ptpn12
1419069_at	RAB guanine nucleotide exchange factor (GEF) 1	Rabgef1
1419946_s_at	RAB2, member RAS oncogene family	Rab2
1449676_at	RAB2, member RAS oncogene family	Rab2
1418623_at	RAB2, member RAS oncogene family	Rab2
1426604_at	ribonuclease L (2', 5'-oligoisoadenylate synthetase-dependent)	Rnasel
1418337_at	ribose 5-phosphate isomerase A	Rpia
1437246_x_at	ribosomal protein S6 /// similar to 40S ribosomal protein S6 (Phosphoprotein NP33) /// similar to 40S ribosomal protein S6 (Phosphoprotein NP33)	Rps6 /// LOC214738 ///

		LOC384383
1439444_x_at	RIKEN cDNA 1110014C03 gene	1110014C03Rik
1454688_x_at	RIKEN cDNA 1110014C03 gene	1110014C03Rik
1439462_x_at	RIKEN cDNA 1110014C03 gene	1110014C03Rik
1428657_at	RIKEN cDNA 1110037N09 gene	1110037N09Rik
1420809_a_at	RIKEN cDNA 1500003O03 gene	1500003O03Rik
1433867_at	RIKEN cDNA 1810030O07 gene	1810030O07Rik
1429411_a_at	RIKEN cDNA 1810057B09 gene	1810057B09Rik
1456393_at	RIKEN cDNA 2310002J21 gene	2310002J21Rik
1437454_a_at	RIKEN cDNA 2310042M24 gene	2310042M24Rik
1417222_a_at	RIKEN cDNA 2310075C12 gene	2310075C12Rik
1452167_at	RIKEN cDNA 2810407C02 gene	2810407C02Rik
1426986_at	RIKEN cDNA 2810485I05 gene	2810485I05Rik
1429159_at	RIKEN cDNA 4631408O11 gene	4631408O11Rik
1418173_at	RIKEN cDNA 4631426H08 gene	4631426H08Rik
1451223_a_at	RIKEN cDNA 4632412E09 gene	4632412E09Rik
1418997_at	RIKEN cDNA 4930469P12 gene /// LOC434102	4930469P12Rik /// LOC434102
1456582_x_at	RIKEN cDNA 5230400G24 gene	5230400G24Rik
1440831_at	RIKEN cDNA 6230421P05 gene	6230421P05Rik
1426355_a_at	RIKEN cDNA 6330578E17 gene	6330578E17Rik
1419977_s_at	RIKEN cDNA 9530027K23 gene	9530027K23Rik
1427985_at	RIKEN cDNA 9630042H07 gene	9630042H07Rik
1433718_a_at	RIKEN cDNA E430007M08 gene	E430007M08Rik
1454725_at	RIKEN cDNA G430041M01 gene	G430041M01Rik
1448434_at	ring finger protein 103	Rnf103
1423740_a_at	RNA binding motif protein 10	Rbm10
1420982_at	RNA-binding region (RNP1, RRM) containing 2	Rnpc2
1437461_s_at	RNA-binding region (RNP1, RRM) containing 3	Rnpc3

1437995_x_at	septin 7	7-Sep
1418422_at	serine (or cysteine) proteinase inhibitor, clade B, member 9g	Serpinb 9g
1419913_at	Serine/threonine kinase receptor associated protein	Strap
1416041_at	serum/glucocorticoid regulated kinase	Sgk
1416862_at	signal transducing adaptor molecule (SH3 domain and ITAM motif) 1	Stam
1439433_a_at	solute carrier family 35 (UDP-galactose transporter), member 2	Slc35a2
1452281_at	Son of sevenless homolog 2 (Drosophila)	Sos2
1417300_at	sphingomyelin phosphodiesterase, acid-like 3B	Smpdl3b
1436809_a_at	spindlin	Spin
1455899_x_at	suppressor of cytokine signaling 3	Socs3
1420175_at	Tax1 (human T-cell leukemia virus type I) binding protein 1	Tax1bp1
1431332_a_at	telomeric repeat binding factor 1	Terf1
1415908_at	testis-specific protein, Y-encoded-like 1	Tspyl1
1422781_at	toll-like receptor 3	Tlr3
1437729_at	Transcribed locus, strongly similar to XP_521837.1 similar to 60S ribosomal protein L27a [Pan troglodytes]	
1436392_s_at	transcription factor AP-2, gamma	Tcfap2c
1424030_at	transcription factor CP2-like 2	Tcfcp2l2
1449671_at	Transmembrane 7 superfamily member 1	Tm7sf1
1419918_at	transmembrane emp24 protein transport domain containing 7	Tmed7
1435064_a_at	transmembrane protein 27	Tmem27
1428586_at	transmembrane protein 41B	Tmem41 b
1424275_s_at	tripartite motif-containing 41	Trim41
1425562_s_at	tRNA nucleotidyl transferase, CCA-adding, 1	Trnt1
1439005_x_at	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Ywhaz
1437714_x_at	Ubiquitin specific protease 14	Usp14
1452011_a_at	UDP-glucuronate decarboxylase 1	Uxs1
1456292_a_at	vimentin	Vim
1457285_at	zinc finger protein 187	Zfp187
1419181_at	zinc finger protein 326	Zfp326
1427590_at	zinc finger protein 39	Zfp39
1449691_at	Zinc finger protein 644	Zfp644
1455930_at		
1447977_x_at		
1436213_a_at		
1419997_at		
AFFX-DapX-M_at		
AFFX-ThrX-M_at		
AFFX-r2-Bs-phe-3_at		
1455602_x_at		
1454794_at		
1436214_at		
1423194_at		
1438855_x_at		
1425514_at		

References

1. Lind, T. Would more calories per day keep low birthweight at bay? Lancet, 1984; 1: 501-2.

. Ravelli, G.P., Z.A. Stein, and M.W. Susser. Obesity in young men after famine exposure in utero and early infancy. N Engl J Med, 1976; 295: 349-53.

3. Roseboom, T.J., J.H. van der Meulen, A.C. Ravelli, C. Osmond, D.J. Barker, and O.P. Bleker. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Twin Res, 2001; 4: 293-8.

4. Neugebauer, R., H.W. Hoek, and E. Susser. Prenatal exposure to wartime famine and development of antisocial personality disorder in early adulthood. Jama, 1999; 282: 455-62.

5. Roseboom, T.J., J.H. van der Meulen, C. Osmond, D.J. Barker, A.C. Ravelli, and O.P. Bleker. Plasma lipid profiles in adults after prenatal exposure to the Dutch famine. Am J Clin Nutr, 2000; 72: 1101-6.

6. Godfrey, K.M. Maternal regulation of fetal development and health in adult life. Eur J Obstet Gynecol Reprod Biol, 1998; 78: 141-50.

7. Barker, D.J. and C. Osmond. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet, 1986; 1: 1077-81.

8. Barker, D.J., P.D. Winter, C. Osmond, B. Margetts, and S.J. Simmonds. Weight in infancy and death from ischaemic heart disease. Lancet, 1989; 2: 577-80.

9. Hsueh, A.M., C.E. Agustin, and B.F. Chow. Growth of young rats after differential manipulation of maternal diet. J Nutr, 1967; 91: 195-200.

10. Downs, K.M. and T. Davies. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development, 1993; 118: 1255-66.

11. Wright, G.W. and R.M. Simon. A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics, 2003; 19: 2448-55.

12. Roseboom, T.J., J.H. van der Meulen, A.C. Ravelli, C. Osmond, D.J. Barker, and O.P. Bleker. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Mol Cell Endocrinol, 2001; 185: 93-8.

13. Stein, A.D., P.A. Zybert, M. van de Bor, and L.H. Lumey. Intrauterine famine exposure and body proportions at birth: the Dutch Hunger Winter. Int J Epidemiol, 2004; 33: 831-6.

14. Stein, Z. and M. Susser. The Dutch famine, 1944-1945, and the reproductive process. II. Interrelations of caloric rations and six indices at birth. Pediatr Res, 1975; 9: 76-83.

15. Lopuhaa, C.E., T.J. Roseboom, C. Osmond, D.J. Barker, A.C. Ravelli, O.P. Bleker, J.S. van der Zee, and J.H. van der Meulen. Atopy, lung function, and obstructive airways disease after prenatal exposure to famine. Thorax, 2000; 55: 555-61.

16. Painter, R.C., T.J. Roseboom, G.A. van Montfrans, P.M. Bossuyt, R.T. Krediet, C. Osmond, D.J. Barker, and O.P. Bleker. Microalbuminuria in adults after prenatal exposure to the Dutch famine. J Am Soc Nephrol, 2005; 16: 189-94.

17. Barker, D.J. and C. Osmond. Diet and coronary heart disease in England and Wales during and after the second world war. J Epidemiol Community Health, 1986; 40: 37-44.

18. Barker, D.J. Intrauterine programming of adult disease. Mol Med Today, 1995; 1: 418-23.

19. Barker, D.J. and P.M. Clark. Fetal undernutrition and disease in later life. Rev Reprod, 1997; 2: 105-12.

20. Winick, M. and A. Noble. Quantitative changes in ribonucleic acids and protein during normal growth of rat placenta. Nature, 1966; 212: 34-5.

21. Heasman, L., L. Clarke, K. Firth, T. Stephenson, and M.E. Symonds. Influence of restricted maternal nutrition in early to mid gestation on placental and fetal development at term in sheep. Pediatr Res, 1998; 44: 546-51.

22. Edwards, L.J., G. Simonetta, J.A. Owens, J.S. Robinson, and I.C. McMillen. Restriction of placental and fetal growth in sheep alters fetal blood pressure responses to angiotensin II and captopril. J Physiol, 1999; 515 (Pt 3): 897-904.

23. Dodic, M., R. Baird, V. Hantzis, I. Koukoulas, K. Moritz, A. Peers, and E.M. Wintour. Organs/systems potentially involved in one model of programmed hypertension in sheep. Clin Exp Pharmacol Physiol, 2001; 28: 952-6.

24. Perry, V.E., S.T. Norman, J.A. Owen, R.C. Daniel, and N. Phillips. Low dietary protein during early pregnancy alters bovine placental development. Anim Reprod Sci, 1999; 55: 13-21.

25. Langley, S.C. and A.A. Jackson. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond), 1994; 86: 217-22; discussion 121.

26. Woods, L.L., D.A. Weeks, and R. Rasch. Hypertension after neonatal uninephrectomy in rats precedes glomerular damage. Hypertension, 2001; 38: 337-42.

27. Langley-Evans, S.C., G.J. Phillips, R. Benediktsson, D.S. Gardner, C.R. Edwards, A.A. Jackson, and J.R. Seckl. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta, 1996; 17: 169-72.

28. Huizinga, C.T., C.B. Oudejans, R.A. Steiner, D.K. Clifton, and H.A. Delemarre-van de Waal. Effects of intrauterine and early postnatal growth restriction on hypothalamic somatostatin gene expression in the rat. Pediatr Res, 2000; 48: 815-20.

29. Perez-Torrero, E., C. Torrero, and M. Salas. Effects of perinatal undernourishment on neuronal development of the facial motor nucleus in the rat. Brain Res, 2001; 905: 54-62.

30. Gheorghe, C., S. Mohan, and L.D. Longo. Gene expression patterns in the developing murine placenta. J Soc Gynecol Investig, 2006; 13: 256-62.

31. Ryan, K.M., A.C. Phillips, and K.H. Vousden. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol, 2001; 13: 332-7.

32. Hofmann, T.G., A. Moller, H. Sirma, H. Zentgraf, Y. Taya, W. Droge, H. Will, and M.L. Schmitz. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat Cell Biol, 2002; 4: 1-10.

33. Yoon, K. and R.C. Smart. C/EBPalpha is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes. Mol Cell Biol, 2004; 24: 10650-60.

34. Bulger, M. Hyperacetylated chromatin domains: lessons from heterochromatin. J Biol Chem, 2005; 280: 21689-92.

35. Mao, Z., S. Liu, J. Cai, Z.Z. Huang, and S.C. Lu. Cloning and functional characterization of the 5'-flanking region of human methionine adenosyltransferase 2A gene. Biochem Biophys Res Commun, 1998; 248: 479-84.

36. O'Connor, M.S., A. Safari, D. Liu, J. Qin, and Z. Songyang. The human Rap1 protein complex and modulation of telomere length. J Biol Chem, 2004; 279: 28585-91.

37. Yu, Z., D. Feng, and C. Liang. Pairwise interactions of the six human MCM protein subunits. J Mol Biol, 2004; 340: 1197-206.

38. Hursting, S.D., J.A. Lavigne, D. Berrigan, L.A. Donehower, B.J. Davis, J.M. Phang, J.C. Barrett, and S.N. Perkins. Diet-gene interactions in p53-deficient mice: insulin-like growth factor-1 as a mechanistic target. J Nutr, 2004; 134: 2482S-86S.

39. Nikolich-Zugich, J. and I. Messaoudi. Mice and flies and monkeys too: caloric restriction rejuvenates the aging immune system of non-human primates. Exp Gerontol, 2005; 40: 884-93.

40. Barker, D.J. Fetal origins of coronary heart disease. Bmj, 1995; 311: 171-4.

41. Barker, D.J. In utero programming of chronic disease. Clin Sci (Lond), 1998; 95: 115-28.

42. McMillen, I.C. and J.S. Robinson. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev, 2005; 85: 571-633.

43. Edwards, L.J., C.L. Coulter, M.E. Symonds, and I.C. McMillen. Prenatal undernutrition, glucocorticoids and the programming of adult hypertension. Clin Exp Pharmacol Physiol, 2001; 28: 938-41.

44. Zhang, L. Prenatal hypoxia and cardiac programming. J Soc Gynecol Investig, 2005; 12: 2-13.

45. Longo, L.D. and W.J. Pearce. Fetal cerebrovascular acclimatization responses to high-altitude, long-term hypoxia: a model for prenatal programming of adult disease? Am J Physiol Regul Integr Comp Physiol, 2005; 288: R16-24.

46. Godfrey, K.M. and D.J. Barker. Fetal nutrition and adult disease. Am J Clin Nutr, 2000; 71: 1344S-52S.

47. Gruppuso, P.A., J.M. Boylan, P. Anand, and T.C. Bienieki. Effects of maternal starvation on hepatocyte proliferation in the late gestation fetal rat. Pediatr Res, 2005; 57: 185-91.

48. MacLennan, N.K., S.J. James, S. Melnyk, A. Piroozi, S. Jernigan, J.L. Hsu, S.M. Janke, T.D. Pham, and R.H. Lane. Uteroplacental insufficiency alters DNA

methylation, one-carbon metabolism, and histone acetylation in IUGR rats. Physiol Genomics, 2004; 18: 43-50.

49. Pham, T.D., N.K. MacLennan, C.T. Chiu, G.S. Laksana, J.L. Hsu, and R.H. Lane. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. Am J Physiol Regul Integr Comp Physiol, 2003; 285: R962-70.

Chapter 5

Conclusions and Future Directions

Hypoxic stress responses: setting up long-term consequences

Early placental development occurs in a relatively hypoxic environment[1]. Oxygen tension is thought to regulate important placental functions such as trophoblast differentiation and invasiveness, vascular development, and nutrient trafficking [2]. Later in pregnancy, maternal hypoxia secondary to illness or lifestyle factors, can lead to placental hypo-perfusion and fetal growth restriction [3]. Studies in our laboratory have shown structural changes occur in the placenta in order to cope with hypoxic stress [4, 5]. Other laboratories have shown significant changes in both placental gene expression and placental morphology. We sought to explore further the genetics mechanisms of the hypoxic stress response by harnessing the power of Microarray analysis. By analyzing over twenty thousand genes simultaneously, we were able to identify several key pathways that were triggered by hypoxia.

We first noted that a group of proteases from the granzyme family were up-regulated by hypoxia. Granzymes were first described in the immune system, where they are secreted by natural killer cells and cytotoxic T lymphocytes along with perforin and trigger apoptosis in target cells. Hypoxia has been previously shown to trigger apoptosis in trophoblast cells [6], and increased apoptotic turnover has been implicated as a possible pathophysiological mechanism of pre-eclampsia [1]. Although their role in apoptosis has been studied extensively, granzymes also have a protease activity and have

been shown to degrade extra-cellular matrix proteins and are expressed in the placenta [7]. Trophoblast and Sertoli cells express granzyme B, and its inhibitor PI-9, independent of perforin, and may play a different role in reproductive tissues [8]. An important follow-up question raised by our studies will be to examine the role granzymes play in placental development.

We also observed that several genes involved in the metabolism of reactive oxygen species and DNA methylation were up-regulated. Reactive oxygen species are generated in response to hypoxia, and have been implicated in numerous cellular responses. They are thought to cause several negative consequences including lipid peroxidation, protein modification, DNA damage, etc (add here refs from hypoxia paper)[9]. One of the scavenging mechanisms the cell uses to deal with ROS is the glutathione system. Glutathione (g-glutamylcysteinylglycine, GSH) is a tri-peptide that has a very high reducing power due to its sulfide group. It is synthesized in the cell by GSH synthetase, and is used up or recycled by a complex network of enzymes. Recent studies have shown that, in addition to its anti-oxidant effects, GSH can modulate protein activity and gene expression. One of the major gene classes up-regulated by GSH signaling were genes involved in DNA methylation [10]. DNA methylation has been shown to be one of the primary means of exerting epigenetic regulation. Epigenetic regulation is defined as changes in gene expression that do not require changes in DNA sequence. Through the addition and removal of methyl groups to DNA or histones, entire regions of the genome become silenced or made available for transcription. Our data also demonstrated an up-regulation of genes involved in DNA methylation. In particular we

observed the up-regulation of DNA methyltransferase 3b which is the main enzyme involved in de-novo establishment of methylation patterns.

These data point to a potential chain of events that may have long-term consequences after the fetus is born and matures into an adult. As illustrated in Figure 1 hypoxic stress leads to an increase in reactive oxygen species, which in turn leads to increased glutathione metabolism and an increase in DNA methylases. Aberrant DNA methylation can then lead to long-term changes in key transcripts. Dysregulation in the expression of the imprinted Igfbp2 gene has already been shown to trigger long-term changes in metabolism. The identification of the genes regulated long term by early stress will be a crucial step in understanding and treating this problem.

Protein Deprivation: Placental coping mechanisms

Nutritional deprivation, and in particular protein deprivation, during fetal development has been shown to have numerous negative long-term consequences for the individual. While the physiological sequelae have been well described, little is known about the genetic mechanisms that trigger them. Our study has shown several potential mechanisms that could mediate and explain the observed long term responses.

Protein deprivation triggered an up-regulation in the p53 pathway which inhibits mitosis and cellular growth. It also triggered the up-regulation of apoptosis - related genes and a host of negative regulators of cellular growth and metabolism. As diagramed in Figure 2, the overall picture was one of extreme down-regulation of a number of key cellular pathways, and up-regulation of the pathways promoting cell death. Several

studies have shown that nutritionally deprived babies are born small for gestational age and suffer deficits in their cardiac, hormonal, and metabolic control. The timing of the deprivation appears to be crucial. In adults, the down-regulation of these pathways appears to promote lower cancer rates, lifespan extension and cardio-vascular health, whereas in fetuses the effects are negative, probably because of the disruption of key developmental events.

Studies in the adult have shown that one of the molecular events triggered by caloric restriction is the induction of epigenetic changes. Sir1 for example, is a gene involved in chromatin remodeling, and is associated with the activity of histone deacetylases. Histone acetylation and methylation are important epigenetic modulators of gene expression, and they are regulated by nutritional restriction. In our data set, we observed the up-regulation of Histone Deacetylase 7a, an important mediator of histone and chromatin architecture.

The overall response generated by protein deprivation in the mouse placenta is one of profound down-regulation of important cell processes. Investigating the roles these pathways play will be the key to understanding the mechanism of fetal programming of adult disease.

Deciphering a "unified stress response"

While hypoxic stress and nutritional deprivation appear to be two very different stressors, some common features emerge at the genetic level. As illustrated in Figure 3, the two common features emerge: the triggering of apoptosis and epigenetic changes. The

hypoxic response is characterized by an up-regulation of metabolism, proteolysis, extra-cellular matrix remodeling, and reactive oxygen species metabolism. The response to protein deprivation is one of profound down-regulation of cellular growth and metabolism and up-regulation of the p53 pathway. At the interface between the two stressors reside two crucial biological processes: triggering of apoptosis and epigenetic change.

The apoptotic response to hypoxia appears to be mediated through the granzyme class of proteins. Granzymes act by directly cleaving caspase-3 and triggering the apoptotic cascade. In the case of protein deprivation, the apoptotic signal appears to be an activation of the p53 pathway. The mechanism of action of p53 as an apoptotic trigger is the up-regulation of several pro-apoptotic proteins. Granzymes are rapid inducers of apoptosis, while p53 acts slower. It is possible that the difference in mechanism is due to the length of exposure.

The epigenetic response to hypoxia is the up-regulation of DNA methyltransferase 3b and associated proteins. In response to protein deprivation, we observed the up-regulation of Histone deacetylase 7a and associated proteins. The DNA methyltransferases act at the DNA level, while the histone deacetylases act at the histone level. Once again we see a divergence between the two stress responses. It will be important to determine if these differences are due to the time of exposure or if they constitute truly separate mechanisms.

Future aims: unanswered questions and future studies

While microarray experiments are powerful tools to help point the finger in the direction of certain biological responses, they are not designed to provide definitive answers. A weakness of microarrays is that they merely provide a snapshot of the transcriptome of the cell in a particular state. There is difficulty, however, in ~~to~~ deciphering temporal and causal relationships. The strength of microarray analysis is in its generation of new hypotheses. While the researcher generally possesses some *a priori* expectations of the potential results, often the data ends up directing further study in unexpected directions.

Several mechanistic and crucial questions remain unanswered by our results. The induction of proteins involved in epigenetic changes raises the obvious query: what ~~target~~ sequences to these proteins target? Our list of down-regulated genes provides a good starting point for this enquiry. We plan to examine select genes on that list for methylation status in response to stress. Another crucial question is the extent to which these epigenetic changes persist until adulthood, and ~~do~~ what is their role in mediating the long-term effects of fetal programming? The placenta is an ephemeral organ, discarded at birth. In order to allow for long-term changes, these epigenetic modifications must also occur in the fetus and be preserved into adulthood. In the future, we hope to measure these genes in the fetus, and observe possible changes in adult animals.

While or data suggests mechanisms for these long term effects, careful confirmation will be required *in vitro*. We will use a cell culture model to explore further both the induction of epigenetic genes and the modulation of potential target genes. The

proposed mechanism of induction of these responses is still rather tenuous and tentative, and careful studies are required to refine and confirm our model.

Ultimately, the long-term goal is to understand and hopefully prevent or treat long-term programming of adult disease. Besides the obvious interest in the biological mechanisms involved, there is also a large potential benefit to society. By designing early interventions one may treat the fetus/infant and prevent an illness destined to manifest itself decades later.

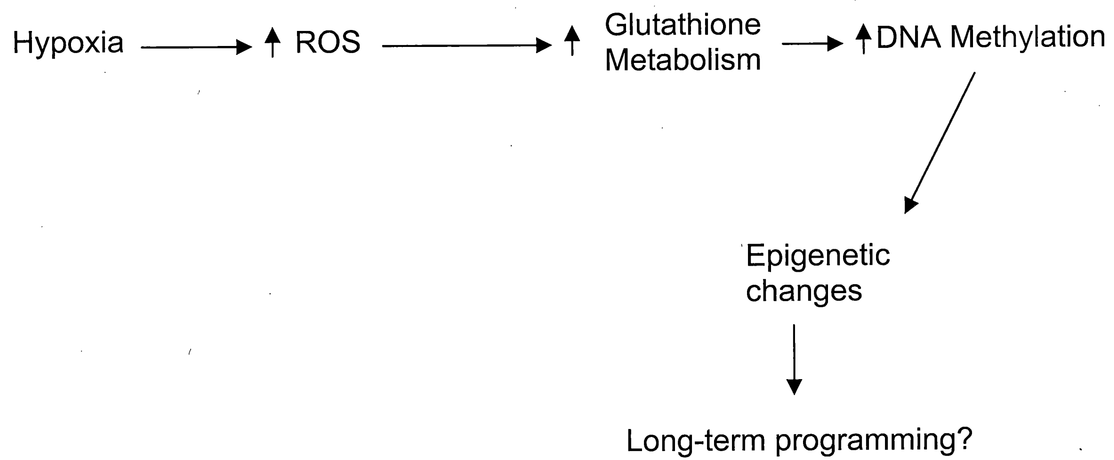

Figure 1: Proposed mechanism of hypoxia-mediated epigenetic changes

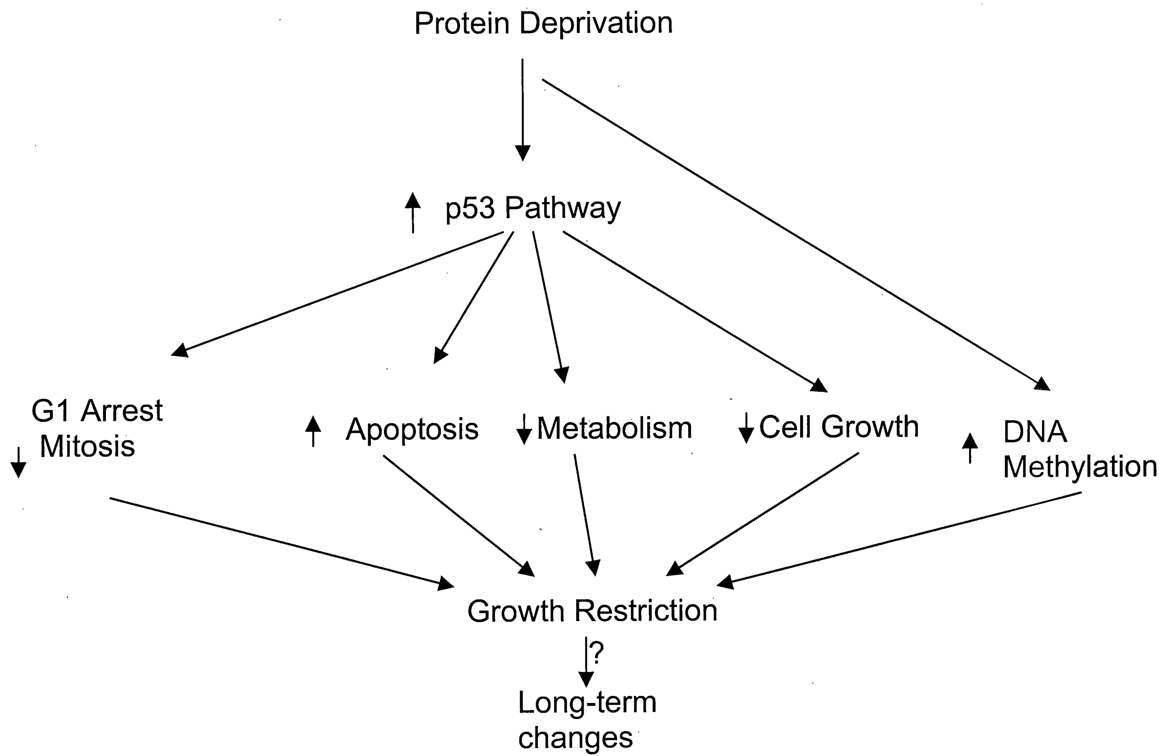

Figure 2: Proposed mechanism of protein restriction induced long-term changes in gene expression

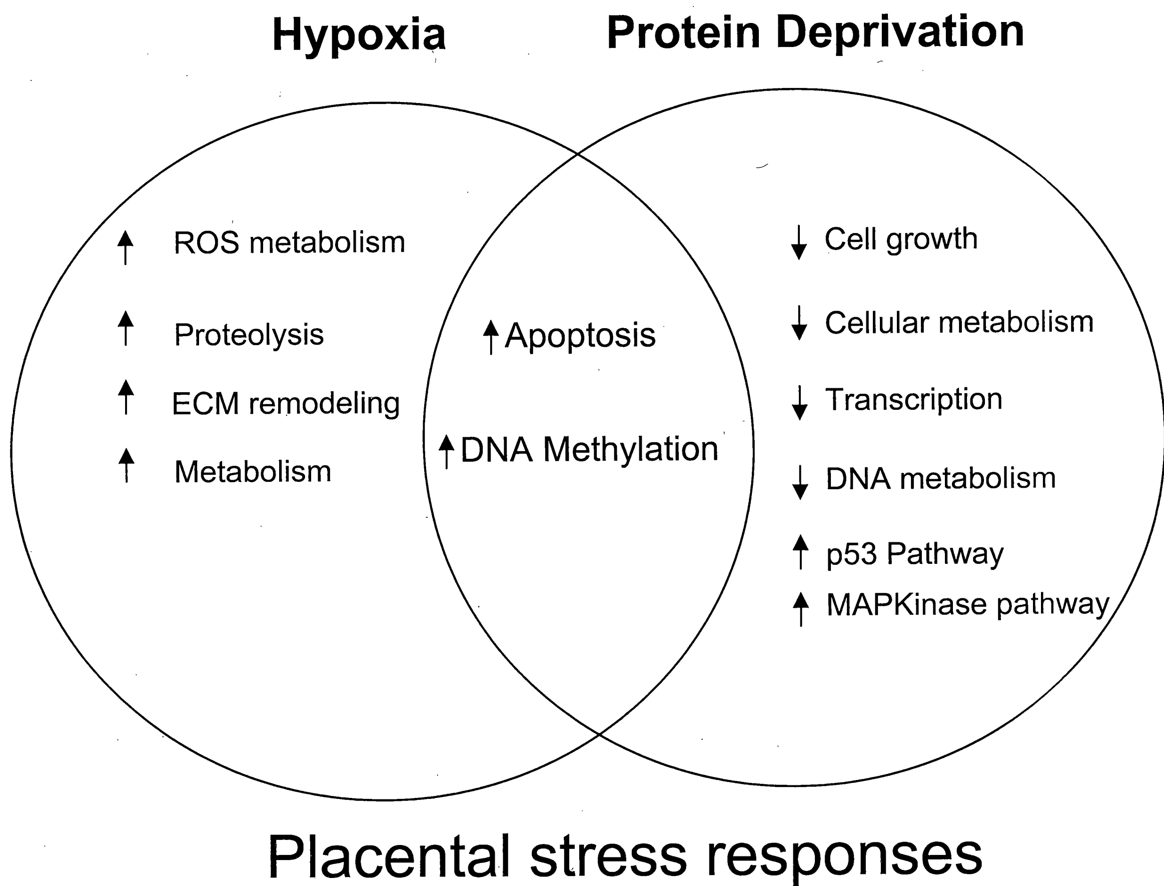

Figure 3: Common patterns of stress response in the mouse placenta

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References

1. Kingdom, J., B. Huppertz, G. Seaward, and P. Kaufmann. Development of the placental villous tree and its consequences for fetal growth. Eur J Obstet Gynecol Reprod Biol, 2000; 92: 35-43.

2. Adelman, D.M., M. Gertsenstein, A. Nagy, M.C. Simon, and E. Maltepe. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev, 2000; 14: 3191-203.

3. Cetin, I., J.M. Foidart, M. Miozzo, T. Raun, T. Jansson, V. Tsatsaris, W. Reik, J. Cross, S. Hauguel-de-Mouzon, N. Illsley, J. Kingdom, and B. Huppertz. Fetal growth restriction: a workshop report. Placenta, 2004; 25: 753-7.

4. Krebs, C., L.D. Longo, and R. Leiser. Term ovine placental vasculature: comparison of sea level and high altitude conditions by corrosion cast and histomorphometry. Placenta, 1997; 18: 43-51.

5. Penninga, L. and L.D. Longo. Ovine placentome morphology: effect of high altitude, long-term hypoxia. Placenta, 1998; 19: 187-93.

6. Levy, R. and D.M. Nelson. To be, or not to be, that is the question. Apoptosis in human trophoblast. Placenta, 2000; 21: 1-13.

7. Allen, M.P. and M. Nilsen-Hamilton. Granzymes D, E, F, and G are regulated through pregnancy and by IL-2 and IL-15 in granulated metrial gland cells. J Immunol, 1998; 161: 2772-9.

8. Hirst, C.E., M.S. Buzza, V.R. Sutton, J.A. Trapani, K.L. Loveland, and P.I. Bird. Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction. Mol Hum Reprod, 2001; 7: 1133-42.

9. Morris, C.J., J.R. Earl, C.W. Trenam, and D.R. Blake. Reactive oxygen species and iron--a dangerous partnership in inflammation. Int J Biochem Cell Biol, 1995; 27: 109-22.

10. Fratelli, M., L.O. Goodwin, U.A. Orom, S. Lombardi, R. Tonelli, M. Mengozzi, and P. Ghezzi. Gene expression profiling reveals a signaling role of glutathione in redox regulation. Proc Natl Acad Sci U S A, 2005; 102: 13998-4003.