The Developmental Origins of Disease/Dysfunction: Environmental Exposures and Epigenetic Mechanisms

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Thanks to those who contributed data…

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• Retha Newbold, NIEHS
• Mike Skinner, Washington State
• Ana Soto, Tufts University
• Moshe Szyf, McGill University
• Cheryl Walker, MD Anderson
• Fred vom Saal, University of Missouri
Overview

- Environment and Disease
- Epigenetics
- Developmental Basis of Disease
  - Fibroids
  - Breast cancer
  - Obesity
  - Fertility
  - Behavior
- Summary
Concept

All complex diseases are the result of:

- Gene-Environment Interactions over Time!

- Recent “epidemics” of chronic diseases like diabetes, childhood asthma, ADHD, obesity... must be due to environmental, dietary and behavioral changes.
Question: If environmental exposures play an important role in disease and dysfunction why has it been so difficult to find/characterize?

Answer: We have been looking in the wrong place (wrong time), and with imprecise measurements of exposure. 

It is also very complex!
Why is it so difficult to define the role of environment in disease in humans?

- Expect effects to be small—mostly functional changes with some specific birth defects...requiring sensitive and specific endpoints.
- Expect effects to be difficult to detect due to human genomic variability and SNPs....requiring a genomic approach.
- Expect effects to be due to multiple chemicals with varying sensitivities and half lives... requiring a mixtures approach.
- Expect effects to be due to “multiple hits”... requiring a lifespan approach.
- Expect in utero exposure to be most sensitive.... requiring a developmental approach.
- Expect some effects to be trans-generational... requiring a multigenerational approach.
- Expect it to be difficult to prove. ...impossible with current technology for exposure assessment and biomarkers of toxicity... requiring improved exposure assessment and biomarkers of exposure and toxicity.
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Epigenetic Alterations: The Molecular “Imprint” Made by Developmental Programming

- Epigenetics... alterations that result in heritable changes in gene expression that do not involve changes in the DNA sequence

- Two types of epigenetic information (marks):
  - Cytosine methylation (DNA)
  - Histone modifications (Protein)

- Epigenetic marks determines the accessibility of the transcription machinery, which transcribes genes into mRNA

- Epigenetic marks control gene expression...on or off

- Epigenetic marks are set during development
DNA Methylation

- CpG islands
  - 1-2% genome
  - Non random
  - 70% Promoter region, first or second exons and first intron
  - Inverse relationship between extent methylation and gene transcription
  - Methylation pattern sculpted during development by DNMTs and demethylases
  - Stable mark...Diagnostic!
- Hypothesis: methylation is dynamic ...sensitive to changes throughout life
DNA Methylation

Reversible gene regulation by DNA methylation

Hypomethylated CpG Island

Hypermethylated CpG Island

Gene activation

Gene silencing
Epigenetic mechanisms of Gene Regulation: Histone Modification:

• N-terminal tails of histones, are subject to various covalent modifications: acetylation, methylation, phosphorylation, ubiquitination.

• Enzymes including histone deacetylase (HDAC), histone acetyltransferasease (HAT), histone methyltransferase (HMTase) are involved.
Environmental factors (Chemicals, diet, drugs, stress, behavior)

Epigenetics (stable but plastic)

Genetic polymorphisms (born with)

Inter-individual variability

Susceptibility to Disease, Toxicants, Drugs, Altered behavior
Developmental Exposures Alter Responses Later in Life

- Diet, Maternal care, Drugs, Toxicants
- Epigenetic Machinery Modulation
- Inter-individual Epigenetic Variation
- Altered Gene Expression Programming
- Phenotypic Variation
- Susceptibility to Disease, Behavior, Sensitivity to Drugs

Szyf tox sci 2007
Epigenetic Basis of Disease!

Environmental Exposures

Normal gene...

Abnormal gene expression

Bad Timing and Amount of Protein

Disease

Pete Myers
Environmental exposures acutely and directly cause altered gene expression via signal transduction pathways and alter the long-term timing of gene expression via epigenetics.
The epigenome is sensitive to and responds to environmental insults during development and throughout life. Development is the most sensitive period.

Epigenetics is a biological mechanism that allows the genome to adapt to altered environments throughout life.

Epigenetic marks are heritable, providing a mechanism for environmental-directed evolution.

Moshe Szyf
“Agents” Shown to Modify the Epigenome

- Methoxychlor
- Vinclozolin
- DES
- Bisphenol A
- Dioxin
- Cigarette Smoke
- Phytoestrogens
- Heavy metals
- Social environment
- High fat diet
- Modulation of one carbon metabolism (SAM/folic acid)
- Valproic Acid (HDAC inhibitor)
- Phenobarbital
Overview

- New Toxicology
- Epigenetics
- Developmental Basis of Disease
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  - Breast Cancer
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- Summary
Fetal Origin of Adult Disease: The Barker Hypothesis

- 1989 David Barker found an inverse relationship between birthweight and death from heart disease in England and Wales.
- Studies confirmed by “Dutch Hunger Winter” when food supplies to occupied Netherlands were cut off by Nazis. Individuals born during this time had high incidence as adults of insulin-resistance.

Fetal Origin of Adult Disease (FEBAD) confirmed for
- Coronary heart disease
- Hypertension
- Type II diabetes

Cheryl Walker
Fetus in utero responds to environmental cues: nutrition, stress.

Depending on in utero conditions it prepares for life....under the assumption that life after birth will match the conditions in utero.

A mismatch leads to increased susceptibility to disease.
Why is the developmental period super sensitive to environmental chemicals? “The Fragile Fetus”

- The developing organism (fetus and neonate) is extremely sensitive to perturbation by chemicals because....
  - Tissues/organs forming
  - Lack of DNA repair
  - No Immune system
  - No Blood/brain barrier
  - Immature Detox enzymes
  - Poor Liver metabolism
  - Epigenetic marks set
Why is the developmental period super sensitive to environmental chemicals?

“The Fragile Fetus”

- The developing organism (fetus and neonate) is extremely sensitive to perturbation by chemicals because...

  Organ development proceeds via an intricately orchestrated, temporal pattern of gene expression that is specific to the developing tissue. As a result, toxic exposures that perturb gene expression may have unique effects in the developing tissue or organ.

- Poor Liver metabolism
- Epigenetic marks set
Developmental Exposures to Environmental Chemicals

- **Teratology**
  - Death
  - Birth Defects
  - Low Birth Weight
  - Functional Changes

Many chemicals will cause all effects depending on the timing and dose!
There is no doubt that development is the most sensitive time for environmental exposures...in animals and humans.

- Exposure to children is higher than adults.
- Low environmentally relevant exposures during development cause “functional changes”.
Developmental Basis of Disease: Disease Focus in Animals

- Reproductive/Endocrine
  - Breast/prostate cancer
  - Endometriosis
  - Polycystic ovary syndrome
  - Fertility
  - Diabetes/metabolic syndrome
  - Puberty
  - Obesity

- Brain/Nervous System
  - Alzheimer's disease
  - Parkinson’s disease
  - ADHD

- Pulmonocardoiovascular
  - Atherosclerosis
  - Asthma
  - Chronic obstructive pulmonary disease
  - Heart disease/hypertension

- Immune/Autoimmune
  - Systemic/tissue specific autoimmune disease
  - Immunosuppression
Developmental Basis of Disease: Environmental Stressor Focus

- Environmental Estrogens
  - Diethylstilbestrol
  - Genistein
  - Bisphenol A
- Tributyl Tin
- Phthalates
- Dioxin/PCBs
- Atrazine
- Smoking/ETS/ Air Pollution
- Methylmercury/Lead/arsenic
- LPS
- Vinclozolin
- Polybrominated diphenyl ethers (PBDE)

NON Mutagenic Effects
Developmental Basis of Disease: Examples

- **Animal Studies**
  - Fibroids
  - Breast cancer
  - Obesity
  - Fertility
  - Behavior
Could it be that susceptibility to uterine fibroids is determined during development and by environmental exposures?
Developmental Basis of Adult Disease: DES as Proof of Principle (Retha Newbold, NIEHS)

• Diethylstilbestrol (DES), a synthetic estrogen, was synthesized by Sir Edward Charles Dodds in 1938.

• DES was widely prescribed from the 1940s thru the 1970s for the treatment of threatened miscarriage.

• Considered safe and effective, also prescribed for normal pregnancies.

• Total # treated pregnancies unknown; worldwide estimates ~ 2-8 million.

• Adverse effects are now well known;
  - Low incidence of vaginal cancer in female offspring.
  - High incidence of reproductive tract dysfunction (male & female offspring).
## Comparative Developmental Effects of Prenatal Exposure to DES in Mice and Humans

<table>
<thead>
<tr>
<th></th>
<th><strong>Male Offspring</strong></th>
<th><strong>Female Offspring</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Functional Changes</strong></td>
<td>Subfertility/Infertility</td>
<td>Subfertility/Infertility</td>
</tr>
<tr>
<td></td>
<td>Decreased Sperm Counts</td>
<td>Poor Repro. Outcome</td>
</tr>
<tr>
<td><strong>Birth Defects</strong></td>
<td>Microphallus &amp; Hypospadias</td>
<td>Oviduct, Uterus, Cervix and Vagina</td>
</tr>
<tr>
<td></td>
<td>Retained Hypoplastic Testes</td>
<td>Paraovarian Cysts of Mesonephric Origin</td>
</tr>
<tr>
<td></td>
<td>Retained Mullerian Remnants (anatomical feminization)</td>
<td></td>
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<tr>
<td><strong>Animal studies mimic Human data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumors</strong></td>
<td>Testicular Tumors</td>
<td>Proliferative Epithelial Lesions in Oviduct</td>
</tr>
<tr>
<td></td>
<td>Tumors in Retained Mullerian Remnants</td>
<td>Vaginal Adenomyosis &amp; Adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>Epididymal Cysts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prostatic Tumors &amp; Inflammation</td>
<td></td>
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Newbold 1999
• Most common tumor of women

• Number 1 indication for hysterectomy in the US, accounting for >2000,000 of these surgeries annually

• Hormone dependent requiring estrogen for growth  
  (Cheryl Walker)
The Developmental Basis of Uterine Leiomyoma: Role of Tumor Suppressor Gene Penetrance

- Tumor: Uterine Leiomyoma
- Tumor Suppressor Gene: TSC2
- Model: Eker rat
- Environmental Agent: Exposure to the xenoestrogen DES

Female rats of the Tsc-2^{EK/+} strain were injected with 10µg DES or vehicle on postnatal days 3, 4, and 5. They were then sacrificed at 5 months and 16 months.

- Carrier (Tsc-2^{EK/+}) + DES
- Carrier (Tsc-2^{EK/+}) + Vehicle
- Wildtype (Tsc-2^{+/+}) + DES
- Wildtype (Tsc-2^{+/+}) + Vehicle
Developmental DES Exposure Increases Tumor Incidence, Multiplicity and Size in Genetically Susceptible Animals.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>N of rats</th>
<th>% Tumor Incidence</th>
<th>Multiplicity (mean no. of tumors/rat)</th>
<th>Size (cm$^3$) Mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Tsc-2^{Ek/+}$</td>
<td>vehicle</td>
<td>28</td>
<td>64</td>
<td>0.82</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>24</td>
<td>92*</td>
<td>1.33*</td>
<td>10.5 ± 2.7*</td>
</tr>
<tr>
<td>$Tsc-2^{+/-}$</td>
<td>vehicle</td>
<td>34</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Developmental reprogramming of estrogen responsiveness

DES

Cook et al. PNAS 2005
Window of Susceptibility to Developmental Programming: When does it Close?

Uterine mesenchyme segregates into 3 layers: inner, middle, outer

Uterine glands present in stroma

- DES d3-5
- DES d10-12
- DES d17-19

Circumference myometrial layer differentiation: d0
- d5
- d15
- ~d35

Longitudinal myometrial layer differentiation

Myometrium maturation, formation of smooth muscle bundles

Puberty
Developmental Re-programming of Estrogen Responsiveness in DES Females

- Target myometrial cells in DES animals hyper-responsive to (low) estrogen levels
- Not observed in liver, which is fully developed in neonates
- Estrogen receptor levels unchanged
- Developmental exposure had reprogrammed estrogen responsiveness
DES Modulates Histone Methylation in Neonatal Uteri

- DES induces global changes in histone methyl marks
- CARM1, an ER coactivator and histone methyltransferase, methyl mark (H3R17) increases $\uparrow$
- EZH2, a methyltransferase inhibited by AKT, methyl mark (H3K27) decreases $\downarrow$
DES Modulates Histone Methylation in Neonatal Uteri

- DES induces global changes in histone methyl marks
- CARM1, an ER coactivator and histone methyltransferase inhibited by AKT, methyl mark (H3K27) decreases

Identification of an “imprint” left by developmental programming such as altered methyl marks may be useful for identification of exposed individuals and as a biomarker for disease susceptibility in adult life.
Environmental agents act on a genetic background.

Environmental exposures can act synergistically with genetic susceptibility factors, in critical pathways to increase susceptibility to disease.

Developmental exposures leave epigenetic marks....
Could it be that other major reproductive diseases, endometriosis, premature menopause, PCOS have their origins in development and are influenced by environmental exposures?
Thought Provoking Idea!

Could it be that Breast cancer susceptibility is determined during development and influenced by environmental exposures?
Gene-Environment Interactions Influence Cancer Risk

- Increased lifetime risk of breast cancer in women born after 1940
- Increased exposure to environmental estrogens
  - Phytoestrogens
  - Oral contraceptives
  - Pesticides
  - Plasticizers

Importance of Environmental Factors on Cancer Risk in BRCA1/2 Ashkenazi Jew mutation carriers

Science, October 2003
USES OF BISPHENOL A IN PRODUCTS
Production Capacity > 6.5 Billion Pounds / Year
Bisphenol A-based polycarbonate is used as:

- a plastic coating for children's teeth to prevent cavities
- as a coating in metal cans to prevent the metal from contact with food contents
- as the plastic in food containers, refrigerator shelving, baby bottles, water bottles, returnable containers for juice, milk and water, micro-wave / oven-ware and eating utensils.
<table>
<thead>
<tr>
<th>EFFECTS IN MICE &amp; RATS</th>
<th>HUMAN HEALTH TRENDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal urethra</td>
<td>Abnormal penis+urethra</td>
</tr>
<tr>
<td>Prostate hyperplasia &amp; cancer</td>
<td>Prostate cancer increase</td>
</tr>
<tr>
<td>Mammary hyperplasia &amp; cancer</td>
<td>Breast cancer increase</td>
</tr>
<tr>
<td>Sperm count decrease</td>
<td>Sperm count decrease</td>
</tr>
<tr>
<td>Early puberty in females</td>
<td>Early sexual maturation</td>
</tr>
<tr>
<td>Hyperactivity/Impaired learning</td>
<td>ADHD</td>
</tr>
<tr>
<td>Abnormal oocytes</td>
<td>Miscarriage*</td>
</tr>
<tr>
<td>Body weight increase</td>
<td>Obesity increase*</td>
</tr>
</tbody>
</table>
Fetal Basis of Breast Cancer: Bisphenol A Research Paradigm

PUMP

preg day 8- birth

0, 25, 250, ng BPA/kg body weight/day

Ana Soto
Exposure to BPA alters overall organization of the fetal Mammary Gland

Accelerated maturation of the stroma, increased accumulation of fat droplets into fat pad adipocytes

Increased number of terminal ends
Increased area subtended by ducts
Increased ductal extension
Exposure to BPA alters overall organization of the fetal Mammary Gland

**CONTROL**

- Increased number of terminal ends
- Increased area subtended by ducts
- Increased ductal extension

**BPA**

Accelerated maturation of the stroma, increased accumulation of fat droplets into fat pad adipocytes

- Increased number of terminal ends
- Increased area subtended by ducts
- Increased ductal extension
Mammary Gland Development:
6 Months

control  25 ng/kg BPA  250 ng/kg BPA
BPA Induces Ductal Hyperplastic Lesions and CIS
Experimental aim: To determine whether fetal exposure to BPA increases mammary cancer risk

0, 2.5, 25, 250, 500 and 1000 μg BPA/kg body weight/day

mate

E1  E9  PND1

Subcarcinogenic NMU exposure

Ana Soto
BPA Increased the Incidence of Tumors After a Subcarcinogenic Dose of NMU
Environmental exposures at environmental levels can cause cancer later in life.

- Breast cancer
- Prostate cancer

In addition to developmental exposures sometimes additional exposures are needed.

- First exposure sensitizes system to second
Could it be that Obesity is determined during development and influenced by environmental exposures?
Obesity: Lessons From Two Mice

1 part per billion DES

100 ppb causes weight loss

Exposure in the womb


--> Obese as adult

Same strain of mice
Same caloric intake
Same activity levels

Pete Myers
Obesity: Lessons From Two Mice

1. Low levels matter
2. High level tests don’t predict low level impacts
3. Fetal exposures alter adult health


Same strain of mice
Same caloric intake
Same activity levels

Pete Myers
Obesogens - Just the Tip of the Iceberg?

- PFOA
- Estradiol
- Genistein
- Phthalates
- DES
- Nicotine
- Organophosphate
- Tributyl Tin
- Bisphenol A
- pesticides
- PCBs?
- PBDEs?
- others?

- What don’t we know yet?
  - Body burdens in population
  - Molecular targets of action beyond RXR-PPARγ
  - Critical windows of exposure
  - How does prenatal exposure alter adult phenotype?
  - Endpoints to study
Thought Provoking Idea!

Could it be that
The effects of developmental exposure
could be transmitted to future
generations
and influence their adult sensitivity
disease?
Fetal Basis and Transgenerational Transmission of Reduced Fertility

Endocrine Disruptor

\[ \text{F0} \leftrightarrow \text{F1} \leftrightarrow \text{F2} \leftrightarrow \text{F3} \leftrightarrow \text{F4} \]

Vinclozolin     Methoxychlor

Anway et al Science 308, 2005
Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility
Developmental Exposure to vinclozolin and 3rd Generation Testicular Morphology

Complete Male Infertility (10%):
100% altered spermatogenesis

Control

Vinclozolin

F3 Generation Males

Skinner, 2005
Not just the testis affected:
Virtually all organs/systems
...for 4 generations!
E16 Testis Transcriptome

Control F1
Control F2
Control F3
Vinclozolin F1
Vinclozolin F2
Vinclozolin F3

Same results from Adults

Michael Skinner
Window of Susceptibility to Methoxychlor Transgenerational Effects

TESTIS DEVELOPMENT

Primordial Germ Cell Re-methylation

Testis Growth

Cord Formation

Mesonephros Cell Migration

Spermatogenesis

Tubule Formation

Sex Determination/Differentiation

Birth

Puberty

Male Fertility

E12  E13  E14  E16  P0  P10  P60

(R = Embryonic Day)

P = Postnatal Day
Concept

- Dosing during the time of primordial germ cell resetting of the epigenetic marks can result in germ cell transmission of a toxic effect.

- What your great-grandfather and grandfather were exposed to can affect your health.
Thought Provoking Idea!

Could it be that Maternal grooming behavior could influence adult sensitivity to stress and disease?
Maternal Behavior in the Rat

M Szyf
Maternal Behavior in the Rat

M Szyf
Maternal behavior programs GR gene activity in the hippocampus which lasts into adulthood.
LG increases histone acetylation and binding of NGFI-A to the hippocampal GR(1γ) promoter

Methylation of CG 16 in [GR(1γ)] promoter inhibits binding of the transcription factor NGFI-A *in vitro* and *in vivo*

Weaver at al., Nature Neuroscience August 2004
Individual differences in stress reactivity of the adult are determined by maternal behavior during infancy.

HIGH LG

Development of Stress Reactivity

LOW LG

Modest Stress Reactivity
Reduced Risk for Disease

Increased Stress Reactivity
Increased Risk for Heart Disease, Type II Diabetes, Alcoholism, Affective Disorders, Brain Aging etc.

M. Szyf
• Social behavior of one subject (mother) can effect epigenetic programming in another subject (child).

• Behavior responds to the environment via epigenetics.
The Developmental Basis of Disease Changes Everything!

- Developmental nutrition and environmental chemical exposures alter gene expression, via epigenetics, leading to functional changes in tissues...leading to increased susceptibility to disease.

- This implies that health outcomes, can be determined by environmental exposures that occurred in early life, possibly decades, before disease becomes apparent.

- There are now numerous examples in animal models of the developmental basis of disease.
  
  Fibroids, Breast Cancer, Prostate Cancer, Fertility
  Obesity, Altered Behavior
The Developmental Basis of Disease Changes Everything!

- This paradigm changes the focus from curing a disease to prevention and intervention strategies to reduce disease incidence.
- It also changes focus from adults to development for the cause of disease.
- Identification of an “imprint” left by developmental programming such as altered methyl marks may be useful for identification of exposed individuals and as a biomarker for disease susceptibility in adult life.
How to Assess Human Risk from Developmental Exposures?

- Problem 1: How to determine exposures during specific windows of exposure
- Problem 2: How to assess functional change
- Problem 3: How to relate functional change to disease later in life
- Problem 4: How to conduct studies for 60 years.

It all comes down to the need for validated biomarkers from animal studies that can be used in humans to indicate potential increase in susceptibility to disease later in life.
Strategy for Assessing Risk

Animal Expt → Human

Internal Dose Met/disp
Epigenetic biomarker validate
Internal Dose
Epigenetic Biomarker

Disease
Same physiology
Disease

Predict
What is Needed?

- Examine more diseases
- Better animal models of disease
- Internal exposure measurements, animals and humans
- Biomarkers of exposure and effect
- Epigenetic biomarkers
- Translation of biomarkers to human studies
- Human studies (dev exposures and biomarkers of effect)
- Lifespan approach
- Mixtures
- Team science...animal/human studies
- Team science...focus on syndromes (phthalate, estrogens)
- New exposures from new sources
Thank You !