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DISRUPTION OF DEVELOPMENT BY ENVIRONMENTAL ESTROGENS: ADULT OBESITY AND METABOLIC DISEASE

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Since the early 1990s (1) evidence has accumulated that chemicals used in a wide range of household products, such as building materials, plastics, cleaning fluids, cosmetics and pesticides have the capacity to disrupt the chemical messengers by which cells communicate (2,3). Exposure to a number of these endocrine disrupting chemicals has been related to obesity and related diseases such as insulin and glucose dysregulation and type 2 diabetes in both experimental animals and epidemiological studies (4-8).

Of the different types of environmental chemicals that disrupt cell signaling, the most well known are those categorized as environmental estrogens. These chemicals have the capacity to bind to classical nuclear estrogen receptors (both the alpha and beta forms) associated with specific genes, and estrogenic chemicals thus act as ligands that initiate transcription of estrogen-responsive genes. Other non-classical estrogen receptors associated with the cell membrane activate signaling enzyme pathways that can rapidly change cell function as well as initiate transcription, and these rapid-response systems greatly amplify extracellular signals (9). However, it is now clear that while generally classified as "estrogenic", the specific responses that each chemical in this class causes may differ from those caused by the most potent endogenous estrogen, estradiol (10). In addition, specific responses caused by estrogenic chemicals and estradiol may vary between tissues in the same species. Estrogenic endocrine disrupting chemicals are thus categorized as "selective estrogen receptor modulators" or SERMs (3). Endocrine disrupting chemicals, for example, organotin antifungal agents, have also been shown to disrupt other signaling systems that play a critical role in the development of obesity, such as the retinoid X receptor (RXR) and peroxisome proliferator activated receptor gamma (PPARγ) (11).

ESTROGENIC CHEMICALS: ORGANIZATIONAL EFFECTS DURING DEVELOPMENT AND ACTIVATIONAL EFFECTS IN ADULTHOOD

Estrogens and other sex hormones regulate the functioning of tissues in adults. These effects occur when the hormone is present and do not occur after the hormone is withdrawn. These are termed "activational" effects. Although, as discussed below, exposure to hormones and environmental chemicals in adulthood can lead to permanent changes in organ function, resulting in disease. However, when exposure occurs during the time in development when cells are differentiating, referred to as "critical periods", estrogens and other hormones typically cause permanent changes termed "organizational" effects. Extensive research is currently directed at elucidating the mechanisms by which genes are "programmed" during cell differentiation under the influence of hormones such as estradiol, as well as endocrine disrupting chemicals. The mechanisms that determine which genes in a cell are able to be transcribed, as well as the level at which transcription occurs, involves "epigenetic" modifications of DNA as well as the associated histone proteins. A schematic depicting "imprinting" mechanisms that determine whether specific genes are silenced or activated is shown in Figure 1 (12,13). There are data indicating that it is via these epigenetic changes that estrogenic endocrine disrupting chemicals "program" gene activity during critical periods in development, with long-term consequences that impact the health status of the individual throughout the remainder of life (14).

ESTROGEN AND OBESITY

The typical view of estrogen is that it is associated with a reduction in food intake and body weight in adults, and the loss of ovarian estrogen secretion associated with menopause in women results in weight gain. However, evidence is accumulating that during critical periods in development, estrogenic chemicals can have unexpected effects on the differentiation of adipocytes as well as postnatal growth (15,16). Specifically, the hypothesis that "programming" of obesity is related to exposure to environmental estrogens during critical periods in organogenesis (17,18) may seem counter-intuitive, as there is considerable experimental evidence that in adult mice estradiol acts via ERα to have an inhibitory effect on adipocyte number and lipogenesis, and removal of estrogen by ovariectomy or via a genetic mutation also causes impaired glucose tolerance and insulin resistance in addition to increased fat mass (19-22). Estrogen has central effects on food consumption and energy expenditure that also contribute to its overall inhibitory effects on adipose deposition in adults. However, a maxim in developmental biology and pediatric medicine is that it is inappropriate to use effects in adults to predict effects during development.

FETAL BASIS OF ADULT DISEASE

The hypothesis that obesity is related to events that occur during early development is know as the "fetal basis of adult disease" hypothesis (7,23). The incidence of metabolic syndrome, which includes obesity, type II diabetes, heart disease and hypertension, has increased dramatically over the last few decades in the USA and many other regions of the world (24,25). The fetal basis of adult disease hypothesis proposes that metabolic syndrome is related to factors that influence growth *in utero* (26).

As indicated above, there is increasing experimental and epidemiological evidence that fetal "programming" of genetic systems is a contributing factor in adult obesity (27). This has led to the hypothesis that epigenetic changes associated with the increased use of manmade chemicals, such as chemicals used in the manufacture of plastic products, may interact with other factors that influence fetal and postnatal growth in contributing to the current obesity epidemic (7,28,29). The hypothesis that epigenetic mechanisms are involved in the etiology of obesity relates to the general issue of developmental plasticity. The hypothesis is that individuals are adapted to function within a restricted range of potential responses throughout life as a consequence of their underlying genetic potential being acted on by environmental factors during the time in tissue differentiation when genetic "programming" occurs (30). Thus, a fetus that develops in a uterine environment in which there is reduced placental blood flow and nutrient transport is thought to develop a "thrifty phenotype", such that the mechanisms mediating weight homeostasis are permanently "programmed" for a lifetime of undernourishment. When exposed to the modern fast foods with excessive calories, these individuals are unable to regulate their body weight, resulting in weight gain (31). The question being posed here is whether during fetal and neonatal life, environmental chemicals are playing a role in "programming" a phenotype that is prone to obesity?

Restriction of placental blood flow is one cause of reduced fetal growth, and light-at-term human babies are at higher risk for subsequent obesity, type 2 diabetes and hypertension (32). There is now convincing evidence in support of the hypothesis that during fetal life environmental factors that influence fetal growth interact with factors that increase the rate of postnatal growth, resulting in obesity and type 2 diabetes. The interaction between prenatal and

postnatal factors is supported by findings that the best predictor of insulin resistance in 8-year-old children is the combination of being light at birth associated with a high postnatal growth velocity. The phenomenon of restricted intrauterine growth followed by accelerated postnatal growth is referred to as "centile crossing" (24). Recent evidence suggests that the age at which the rapid body weight increase occurs during postnatal life is a critical factor in the eventual health status of a person (23).

ESTROGEN AND THE DIFFERENTIATION AND REGULATION OF ADIPOSE TISSUE

Hormones are major regulators of adipose tissue and are critical for adipocyte development and function. An extensive array of hormones and growth factors modulate adipocyte development and activity, including growth hormone, thyroid hormone, catecholamines, glucagon, insulin and insulin-like growth factors, glucocorticoids, and relevant to this proposal, estradiol and thus chemicals with estrogenic activity (18,33,34). In humans, differentiation of preadipocytes into adipocytes begins prior to birth, but the majority of preadipocytes differentiate postnatally. Adipocyte number increases markedly between birth and 18 months of age, then continues to increase more slowly throughout early childhood (35). The developmental sequence by which the adipocyte phenotype arises from undifferentiated connective tissue cells has been described in detail (36). The adipocyte lineage arises from undifferentiated mesenchymal cells, which can also give rise to other connective tissue lineages. Mesenchymal cells become committed to an adipogenic lineage and give rise to preadipocytes, which can remain undifferentiated and quiescent, proliferate but remain undifferentiated, or differentiate as a postmitotic adipocyte (11).

While preadipocytes in mice do not begin to differentiate into adipocytes prior to birth (37), mouse preadipocytes develop from mesenchymal cells and proliferate during fetal life, and, as discussed further below, they express estrogen receptors (15). Mouse preadipocytes continue to proliferate rapidly at birth, but then the majority enters the differentiation pathway neonatally to give rise to postmitotic adipocytes. By approximately 3 weeks of age, the basic adult number of adipocytes has been established in most mouse strains (38). A preadipocyte population still remains in adults and can give rise to new adipocytes at any time during life in both mice and humans (39).

The genes critical for inducing adipocyte differentiation, as well as their temporal sequence of expression during adipocyte differentiation are being actively investigated (40). For example, it is known that CCAAT/enhancer-binding proteins, such as C/EBPα, along with PPARγ play critical roles in adipocyte differentiation from the preadipocyte to the fully functional, postmitotic adipocyte (41), and that the transcription factor CREB plays an important role in regulating these genes (42). In humans, C/EBPα mRNA levels in adipocyte tissue are elevated in those with an obese relative to lean phenotype (43). PPARγ is expressed in adult adipocytes. PPARγ and C/EBPα are regulators of lipogenesis in addition to regulating adipocyte differentiation, and PPARγ activators result in increased fat deposition (41,42,44). Although obesity typically involves adipocyte hypertrophy, adipocyte hyperplasia is also seen in certain types of human obesity, and similar results have been obtained in rodents with various types of obesity resulting from dietary modification or gene knockouts (36,38).

In abdominal fat, mitochondrial glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial step in glycerolipid synthesis (45). Mice deficient in diglyceride acyltransferase (DGAT1) are resistant to diet-induced obesity and have increased insulin and leptin sensitivity

(46). The presence of the enzyme Cyp19 (aromatase) in adipocytes provides a source of intracellular estradiol via aromatization of testosterone, and aromatase activity in tissues is influenced by estrogen, including the estrogenic chemical bisphenol A (BPA) (47,48). In aromatase knockout mice, an increase in adipocyte volume and number was observed (22,49). Lipoprotein lipase (LPL) is a key enzyme in regulating lipids. Adipocytes express lipoprotein lipase, which attaches to the luminal surface of endothelial cells in capillaries. Lipoprotein lipase binds lipid particles such as chylomicrons and very low-density lipoprotein and breaks down the triglyceride core into free fatty acids. This step is rate limiting and necessary for formation of low-density lipoproteins (LDL), high-density lipoproteins (HDL), and free fatty acids from lipid particles. Free fatty acids can cross membranes into cells, while triglycerides cannot. As a result, lipoprotein lipase in capillaries of a tissue, including adipose tissue, is necessary for the uptake of lipid. Adipose tissue with a lower level of endothelial lipoprotein lipase will not uptake lipid as rapidly. Lipoprotein lipase is subject to regulation by estrogen (49). Estradiol was reported to markedly decrease the amounts of lipoprotein lipase mRNA as well as triglyceride accumulation in 3T3-L1 adipocytes (50).

A critical question to consider when postulating potential effects on adipocyte number and subsequent function generated during development is whether such effects would be permanent or transitory and reversible once the stimulus inducing the change in adipocyte is removed? In summary, the fetal period is a time in the mouse when changes in circulating estrogen (either exogenous or endogenous) could result in changes in adipose tissue function during later life, and changes in the methylation pattern of genes is one potential mechanism. This prediction is consistent with evidence from other systems that exposure to sex hormones and estrogenic endocrine disrupting chemicals during fetal life can have latent effects on the functioning of tissues after birth (33,51).

In recent years the focus on obesity has involved the "big two" factors thought to be primarily responsible for the dramatic increase in obesity over the last two decades: reduced physical activity and over consumption of "junk" food associated with food marketing practices (29). However, it seems likely that environmental factors are also involved, and sorting out which factors are most important has not received much attention (7). Thus, few epidemiologic studies have examined the role of environmental estrogens in the human obesity epidemic (5,6), although data from studies with experimental animals as well as cell culture studies indicate that such studies are needed.

Estrogen is known to play an important role in regulating adipose deposition in males, and estrogen regulates key developmental events in adipogenesis (22). Thus, while the factors regulating whether preadipocytes proliferate or differentiate are not well understood, estrogen appears to be one factor involved in their development. Adipose tissue expresses both ERα and ERß. ERα is expressed in adipocytes, preadipocytes and stromal vascular cells, indicating that almost all cells in adipose tissue are potentially estrogen responsive (52,53). Estrogens appear to play a crucial role in establishment of adult adipocyte number, although effects of estrogens on adipose tissue are complex, and may vary with cell type. A number of papers have shown that estradiol increases proliferation in pre-confluent 3T3-L1 preadipocyte cells or in human or rat preadipocytes *in vitro* (54-56). Estradiol treatment of cultured preadipocytes induces increased release of mitogenic substances into the media (57). Adipocyte hyperplasia is a particular problem because the increased adipocyte population appears to make it very difficult to ever overcome the obesity and maintain a normal weight. It is thus possible that during fetal life exposure to estrogenic chemicals may facilitate a particularly intractable type of obesity, and this

may occur via epigenetic "programming" of genes during "critical periods" in development, which result in permanent changes in gene activity (15).

THE ESTROGENIC CHEMICAL BISPHENOL A (BPA) AND OBESITY

BPA is one of the highest volume chemicals in worldwide production, with production capacity exceeding 6-billion pounds per year for use in manufacturing polycarbonate plastic, the resin that lines metal cans, and as an additive in many other types of plastic (58). *A*ll human fetuses that have been examined have measurable blood levels of BPA (59-62), and mean or median levels found in humans are higher than levels in fetal and neonatal mice in response to maternal doses that increase postnatal growth (17,63,64).

Exposure during gestation and lactation to BPA has been shown to result in a wide range of effects observed during postnatal life in mice, rats and a wide range of other vertebrates and invertebrate species (65,66). We initially reported (17), and other studies have confirmed (67-71), that prenatal exposure to very low doses of BPA increases the rate of postnatal growth in mice and rats. In addition, neonatal exposure to a low dose (1 μg/kg/day) of the estrogenic drug diethylstilbestrol (DES) stimulated a subsequent increase in body weight and an increase in body fat in mice (72).

There are a number of genes that are likely to be involved in the effects of estrogenic chemicals such as BPA on adipocyte differentiation and function. The genes critical for inducing adipocyte differentiation, as well as their temporal sequence of expression during adipocyte differentiation are being actively investigated (40,73). We have preliminary evidence that expression of a number of genes is permanently altered as a result of differential fetal growth (based on comparisons of intrauterine growth restricted (IUGR) and macromosic male mice), and some of the same genes are also permanently altered due to developmental exposure to BPA (34).

We are examining expression and the DNA methylation profile of these candidate genes, which are implicated in adipocyte differentiation, function and obesity, such as PPARγ, C/EBPα, LPL, GLUT4, Cyp19 (aromatase), GPAT and DGAT. In rats, developmental exposure to approximately 70-μg/kg/day BPA resulted in up-regulation of a number of genes in abdominal adipocytes, including PPARγ and C/EBPα and LPL (34). In mouse 3T3-L1 cells BPA increased lipoprotein lipase (LPL) activity and triacylglycerol accumulation; BPA resulted in the presence of larger lipid droplets in the differentiated cells (74). Insulin and BPA interacted synergistically to further accelerate these processes. BPA also stimulated an increase in the glucose transporter GLUT4 and glucose uptake into 3T3-F442A adipocytes in cell culture (75). In a separate study up-regulation of GLUT4 increased basal and insulin-induced glucose uptake into adipocytes (76). In addition, low doses of BPA stimulated rapid secretion of insulin in mouse pancreatic ß cells in primary culture through a non-classical, non-genomic estrogen-response system, and the magnitude of the response was the same at equal doses of BPA and estradiol. In contrast, prolonged exposure to a low oral dose of BPA (10 μg/kg/day) resulted in stimulation of insulin secretion in adult mice that was mediated by the classical nuclear estrogen receptors; the prolonged hypersecretion of insulin was followed by insulin resistance (77). In a subsequent study (8) these investigators reported that mice exposed during fetal life to a low (10 μg/kg/day) were heavier at birth relative to controls, and at 6 months of age, males prenatally exposed to BPA displayed glucose intolerance, insulin resistance and altered insulin release from pancreatic β cells compared with control mice. It required exposure to a higher dose of BPA (100 μg/kg/day) to have a subsequent effect on the pregnant mice, that displayed glucose intolerance

and altered insulin sensitivity compared with unexposed controls. Furthermore, 4 months after delivery, mice treated with BPA were heavier than control unexposed mice and had decreased insulin sensitivity and glucose intolerance. This finding stands in contrast to the typical assumption that exposure to chemicals such as BPA in adulthood alter metabolic systems during the time of exposure (activational effects), but that the consequences of exposure are not permanent. This observation suggests that BPA exposure during pregnancy in women may affect body weight and glucose metabolism later in life. Consistent with the prediction that fetuses are more sensitive to environmental chemicals than adults, the permanent effects of BPA on offspring metabolic systems occurred at a dose 10-fold lower than the dose required to cause effects in the adult mother (8).

THE ADULT PHENOTYPE DUE TO INTRAUTERINE GROWTH RESTRICTION (IUGR) IS SIMILAR TO DEVELOPMENTAL EXPOSURE TO BPA

There is extensive epidemiological evidence showing that babies with IUGR who then experience a rapid "catch-up" growth spurt during childhood are at high risk for adult obesity and type 2 diabetes, consistent with the "fetal basis of adult disease" hypothesis (27). Thus, fetal growth rate interacts with childhood growth rate in terms of whether IUGR leads to adult obesity and other metabolic diseases. We have developed a novel crowded uterine horn mouse model that results in siblings that range from growth restricted to macrosomic due to differences in placental blood flow based on location in the crowded uterus (Figure 2). Importantly, the IUGR mice experience a rapid period of catch-up growth: IUGR mice experience about a 90% increase in body weight, while macrosomic males have about a 30% increase in body weight during the first week after weaning. Adult IUGR male mice show marked similarities to IUGR humans in terms of glucose intolerance and elevated insulin as well as an increase in total abdominal fat weight (78). Importantly, while both IUGR and macrosomic males remained significantly heavier than male mice with a median body weight at birth, we have found significant differences in adipocyte gene expression and number in adult male gonadal fat. There is thus a markedly different etiology of obesity in these two overweight sub-populations of mice relating to differences in both fetal and post-weaning growth (B. Coe, unpublished observation).

As noted above, our initial studies show that there are some interesting parallels between the effect of perinatal exposure to BPA in a preliminary study and the consequence of a mouse pup being IUGR: in each case there is a greater rate of weight gain during the week following weaning, and in adulthood there are significantly fewer adipocytes, although the adipocytes are significantly larger (B. Coe, unpublished observation). BPA exposed developing mouse pups thus appears to have traits similar to those of IUGR pups. This is interesting in that a recent occupational study from China reported data suggesting that maternal exposure to BPA was related to IUGR (79). In addition, there are similarities in gonadal adipocyte gene expression in comparisons of IUGR vs. macrosomic animals and the effects of BPA vs. controls, since similar to IUGR, BPA elevates PPARγ, C/EBPα and LPL in abdominal adipocytes (34).

THE ENDOCRINE DISRUPTOR TRIBUTYLTIN AND OBESITY

Recent publications have implicated some other environmental chemicals with changes in adipocyte function. For example, organotin compounds were used for many years to protect the bottom of boats, and these persistent compounds remain a health problem even though their use has been restricted in recent years. In addition, organotin compounds are used as stabilizers in

polyvinylchloride (PVC) plastic, which is used to manufacture the pipes that carry water into homes, and organotin compounds leach out of PVC into water (80-82).

Tributyltin (TBT) is an organotin compound and is an endocrine disrupting chemical that results in imposex in gastropod mollusks. This is a condition in which abnormal masculinization of females occurs due to the inhibition of the estrogen-synthesizing enzyme aromatase, which results in an increase in testosterone; testosterone is the substrate which is aromatized by aromatase to form estradiol-17ß (83). TBT also inhibits aromatase activity in human granulose cells in culture (84). TBT is a ligand for PPARγ as well as for retinoic X receptors (RXRs), which, along with estrogen receptors and PPARs, are members of the nuclear receptor superfamily. In mice, treatment of pregnant females with TBT stimulated adipocyte differentiation and increased fat mass in offspring (number and volume of adipocytes was not reported). This finding is thus consistent with other findings described above regarding PPARγ activation during adipogenesis, and adds to other evidence that activation of RXRs are also involved (11,85).

CONCLUSIONS

At this time there is limited human data relating obesity with environmental chemicals, and specifically environmental chemicals that are estrogenic or otherwise disrupt estrogen homeostasis. However, there has been an increase in research relating environmental chemicals with obesity, insulin and glucose dysregulation, and type 2 diabetes. Findings from these studies have led to an increased awareness that energy expenditure and components of a person's diet, while important, likely do not explain the rate of increase in obesity that has been documented over the last two decades (28,29). While the contribution of environmental chemicals to the obesity epidemic remains a largely unexamined issue, the dramatic increase in the incidence of obesity has occurred in parallel with a dramatic increase in the use of plastic. Plastic materials contain many types of endocrine disrupting compounds in addition to BPA. The animal experiments showing a relationship between accelerated postnatal growth, altered insulin secretion and glucose sensitivity due to developmental exposure to daily doses of BPA within the range of human exposure provide a strong argument for further research into the possibility that developmental exposure to BPA, as well as other endocrine disrupting chemicals, is contributing to the development of obesity later in life.

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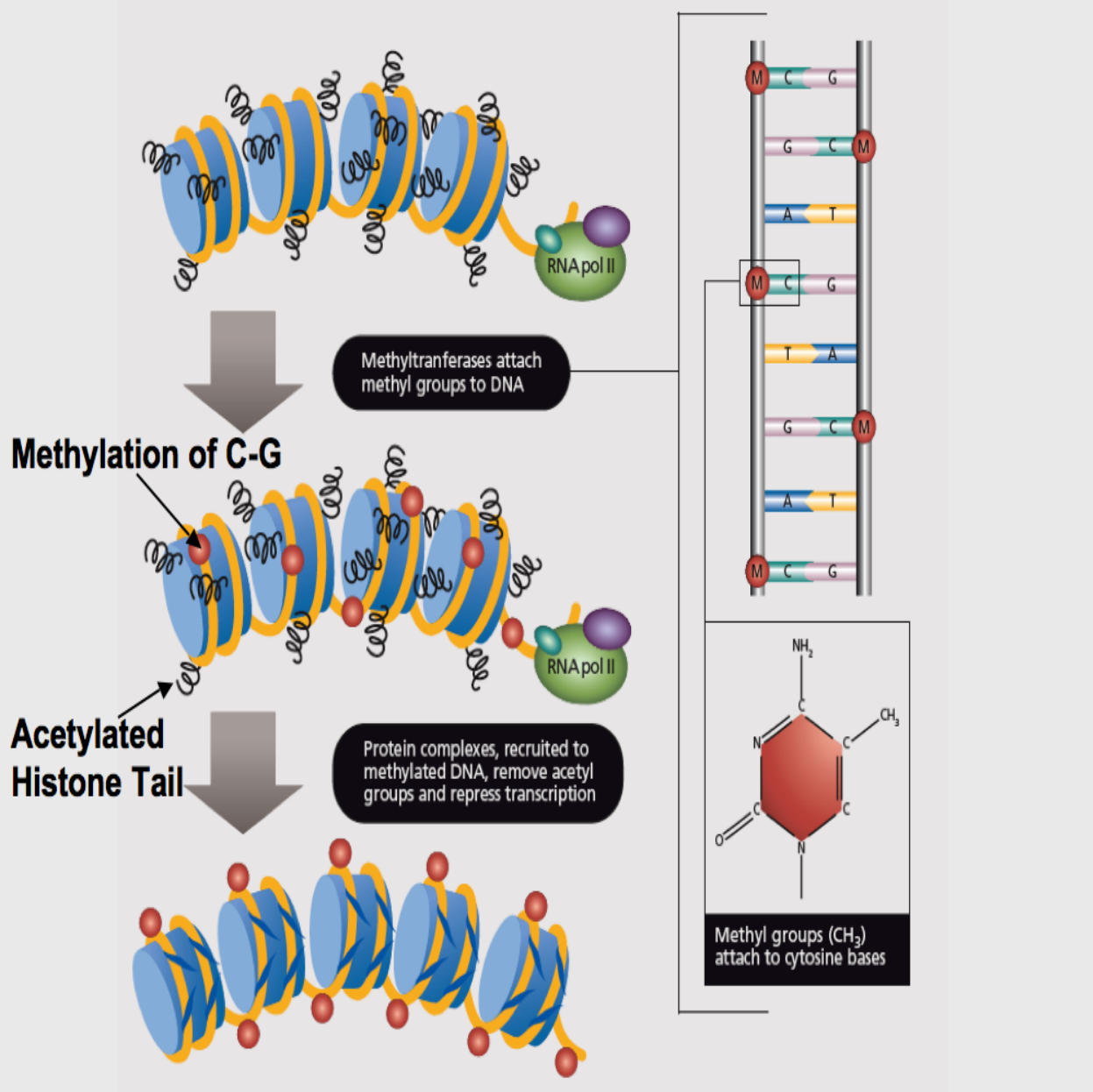
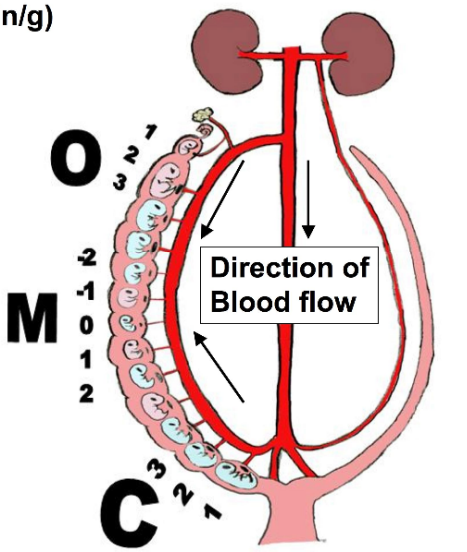
FIGURE 1



Schematic diagram showing the "epigenetic" chemical modifications of histone proteins by removal of acetyl groups and cytosine bases by addition of methyl groups that result in repression of transcription. Epigenetic changes that occur early in development are transmitted to daughter cells during mitosis and thus can permanently alter gene transcription in tissues (Modified from Ref 13).

FIGURE 2

Uterine Position	Placental Blood Flow (ml/min/g)
O 1	31.5
O 2	33.1
O 3	20.6
M 2	20.8
M 1	9.3
M 0	18.8
M 1	27.3
M 2	29.7
C 3	31.3
C 2	43.5
C 1	36.6



Blood flow (indicated by arrows) into the loop uterine artery is bi-directional from both the ovarian (O) and cervical (C) ends of the uterine horn, which leads to greater placental blood flow at the ends relative to the middle (M) of each uterine horn. The data shown are placental blood flow measurements taken from a hemi-ovariectomized pregnant female CD-1 mouse on gestation day 18; the female was injected with radiolabelled microspheres to measure blood flow (86). Removing the left ovary prior to pregnancy results in double the number of oocytes being ovulated from the remaining right ovary and crowding in the right uterine horn, which is separate from the left uterine horn in mice and rats.