How nutrition and the maternal microbiota shape the neonatal immune system

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Abstract:
Mucosal surfaces of mammals are densely colonized with microbes commonly referred to as the commensal microbiota. It is believed that the fetus in utero is sterile and colonization with microbes starts only after birth. Nevertheless, the unborn fetus is exposed to a multitude of metabolites originating from the commensal microbiota of the mother that reach systemic sites of the maternal body. The intestinal microbiota is strongly personalized and influenced by the environment including nutrition as one of the main shaping factors. On the other hand, members of the microbiota can metabolize dietary components that can reach the maternal host and thus the offspring. The complex interplay between nutrition and microbiota of the mother and its effect on offspring immunity is the basis for this Review.

Introduction

From the point of view of immunologists who study mammalian systems, it is easy to forget that viviparity (that is, the birth of live offspring) has appeared independently many times in the animal kingdom during evolution [1]. This speaks for the clear advantages in protecting the developing animal to guard its nutrition, nitrogen balance, gaseous exchange and very existence. Mammalian evolution provides protection in early postnatal life with nutritional and immune support through lactation. The zenith of maternal succor has been reached in the eutherian mammals. These are characterized by a placenta, which serves as a highly adapted organ for molecular exchange between mother and fetus. Interestingly, placentation coevolved with epigenetic imprinting – the DNA modification system that marks certain loci for selective expression in the offspring, depending on whether they have been derived from the male or female parent. The fact that this special genetic control mechanism has evolved to regulate placental function and resource allocation between fetus and mother speaks to the delicate intimacy of having two individuals in such close contact. Despite its inevitable biological problems, placentation has permitted the rich molecular exchange between mother and fetus that has enabled the evolution of a human nervous system with advanced cognitive functions.

The mammalian fetus is supported during development by the umbilical cord [2], through which pass almost all nutrients, respiratory gases, excretion products and xenobiotics. It has been recently realized that molecules from the intestinal microbiota are also an important part of the placental molecular exchange. After birth, the defining purpose of lactation is to support nutrition of the newborn. However, to an extent that varies depending on the mammalian species concerned, lactation also serves as a route of immunoglobulin, macromolecular and xenobiotic uptake and has strong effects on modulating the incoming microbiota of the offspring [3].

In other words, as the placental mammal develops from one cell into an organism of about 10^{13} cells and undergoes the challenges of postnatal adaptation, it is exquisitely dependent on molecular exchange of different sorts with its mother. The requirement for a diet sufficient in calories and balanced in micronutrients (vitamins and minerals) was established mainly from experiments with animals in the first half of the twentieth century A [2]. The importance of maternofetal molecular exchange is generally understood by the lay public to be critical to healthy fetal development: pregnant women optimize their nutrition, avoid alcohol and (after the horrors of thalidomide-induced phocomelia) all non-essential medications.

The intestinal microbiota modulates essentially all aspects of these processes and

contributes its own molecular signature to the tissues and the fetus of the pregnant mother. Indeed, the inherent microbial metagenomic diversity greatly expands the metabolic capacity of the host. Classical organic chemistry studies of selected molecules, modern wider-ranging metabolomics techniques and isotope transfer studies all show that a diverse range of microbial derived molecules pervasively penetrate host tissues. This Review sets out to examine the relative roles of nutrition and microbial metabolites in materno-fetal molecular exchange and how these shape healthy immune system development. The evidence will be mainly drawn from mouse models (because the data largely depend on our ability to experimentally manipulate diet and environmental factors including the colonization status). We will also describe the comparisons with humans in placentation, fetal development, postnatal lactation and immunoglobulin transfer, and hypothesize how these might affect the human host-microbial interactions in early life.

The fetal basis for adult disease

To emphasise how far events in fetal life shape the long-term health of the offspring, we need to start with the effects of maternal nutrition on development and systemic disease [4]. Nutrition was originally shown to be a vital determinant of fetal growth and development from farm animal experiments dating from the first half of the last century [5] and from studies on humans in the tragic conditions of malnutrition in Europe at the end of the second World War [4].

There is abundant evidence that nutritional and other molecular events in fetal and neonatal life lay a foundation for future health, including immune system function. For example, adult abdominal adiposity (itself predisposing to cardiovascular disease and type 2 diabetes) is associated with prior poor intrauterine fetal growth. Lower birth weight has been linked to increased susceptibility in later life to osteoporosis, depression and prostate tumours [6-10]. This epidemiological evidence implies that the adaptation to reduced nutrition in early life has profound long-term consequences. One mechanism for this — discussed in more detail later — is that the fetal epigenetic reprogramming that attempts to salvage fetal growth in the face of an inadequate macronutrient supply from the mother, persists into adult life when nutritional conditions are better. Malnutrition also decreases the levels of maternal antibody transferred to the fetus via the placental neonatal immunoglobulin receptor (FcN) with long term effects on disease susceptibility, B cell repertoire development, allergy and autoimmunity [11,12].

The concept of a fetal basis of adult disease also extends to consumption of xenobiotics. The consequences of exposure to the synthetic non-steroidal estrogen diethylstilbestrol prescribed to pregnant women with the intention of avoiding miscarriage are a disastrous example of this. The consequences of diethylstilbestrol on male and female infertility and reproductive tract tumours only became apparent in adult life, or even in the next-generation offspring of those who were exposed in utero [13]. These are extreme examples, although it should be noted that the intestinal microbiota has powerful effects on modulating the toxicity of many environmental and pharmaceutical xenobiotics and heavy metals [14,15].

Maternal support of fetal immune development

Molecular transfer of nutrients and microbial molecules in early life immune development. Immunity is no exception to the powerful effects of the *in utero* environment. Intrauterine

growth retardation is strongly associated with susceptibility to postnatal infectious disease with effects seen both in children and young adults. Malnutrition in utero impairs immune function through both direct and indirect mechanisms (**Figure 1**). For example, the loss of macronutrients and micronutrients directly affects leukocyte development in the fetus. In addition, maternal malnutrition leads to stress responses in mother and fetus that directly affect placental function and fetal immune development. Malnutrition also leads to maternal immunosuppression, which decreases the availability of maternal immunoglobulin for uptake into the fetus and renders the mother susceptible to opportunistic or manifest infections. Any increase in systemic exposure to microbes will further increase the maternal stress responses through the hypothalamo-pituitary-adrenal (HPA) axis [16].

There are several examples that illustrate how the direct limitation of in utero nutrient levels can affec immunity in neonates. Insufficient supply of vitamin A results in impaired fetal differentiation of B lineage cells, including the B1a and B1b subsets that express antibodies that are responsible for the early phase of protection against pathogens [17]. In addition, maternal retinoids influence the development of fetal lymphoid tissue inducer (LTi) cells and subsequently the size of secondary lymphoid organs in the offspring [18]. Retinoic acid is also required for thymic development and myeloid cell differentiation. As a final example, a deficient zinc supply limits the size of the thymus and spleen, and is associated with deficient B cell and T cell function partly because of stress-induced corticosteroids [19].

Stress responses through the HPA axis can be generated either directly because of protein calorie malnutrition, or indirectly, as malnutrition leads to immunosuppression, poor intestinal barrier function and increased susceptibility to infection. In a healthy pregnancy, the fetus is protected from maternal glucocorticoid exposure because of 11-ß dehydroxylase activity in the placenta, which converts glucocorticoids into non-active metabolites [20], but malnutrition results in reduced placental dehydroxylase levels. Despite this, the effects of maternally delivered glucocorticoids (which suppress the fetal HPA) are much less than direct stimulation and activation of the fetal HPA through corticosteroids present within in the fetus. This has been experimentally carried out with lipopolysaccharide administration or the use of CpG to mimic the effects of bacterial or viral infections [16]. Such manipulations have clear durable effects on many aspects of immunity in the offspring including reduced lymphocyte proliferation, antibody responsiveness and NK activity. There is also a durable resetting of the offspring's HPA activity, resulting in relatively increased stress responses in later life (reviewed in [16]). These effects are likely due to epigenetic programming: a topic that is discussed further below.

Therefore, it is clear that maternal nutrition is vital for the healthy development of the infant immune system. This sets the scene to ask how far the microbiota (which is well known to increase the efficiency of energy extraction from the food [21], provide vitamins and metabolise xenobiotics [22]) contributes to the general nutritional state in pregnancy.

Effects of intestinal microbes on maternal nutrition.

Intestinal microbes can increase the energy harvest from the diet by digesting complex carbohydrates (such as the constituents of plant cell walls) that are resistant to mammalian enzymes [21]. Clearly, the microbiota cannot itself serve as a substitute for a healthy diet, as when the diet is inadequate there is insufficient energy to salvage. However, microbial metabolic capability is required for vitamin synthesis and to generate the short-chain fatty acids that sustain epithelial integrity [23] and regulatory T cell differentiation in the mother [24-26]. The evidence for this comes from specific supplementation and from studying germ-free

animals, which require fortified diets. This is especially true for vitamin K; if animals that are raised in germ-free conditions are not supplemented with this vitamin, the synthesis of host clotting factors is impaired, leading to a bleeding diathesis. B group vitamins, including folate (also known as vitamin B9) and vitamin B12, are also synthesized by the microbiota but not by the host. The synthesis of these vitamins mostly occurs in the large intestine where uptake into the host is more restricted than in the small intestine [27].

As maintenance of the germ-free state depends on rigorous sterilization of the food, normally through prolonged autoclaving of chow, the diets are generally heavily fortified with vitamins to avoid potential micronutrient deficiencies in the animals. Therefore, determining exactly where the boundaries of the microbial contribution to the maternal nutritional state lie requires chemically defined rigorously sterilized diets. This is not as straightforward as it sounds, because doses of irradiation that are used to sterilize chemically defined diets also reduce the vitamin availability of the diet through radiochemical effects [28].

The proportion of micronutrients that are derived from the microbiota in mice depends on the extent of coprophagia **[G]**, which is seen only in psychiatric cases in humans. Nevertheless, although most of the vitamins that are newly synthesized will be shed in the feces, there is evidence even in humans that some B_{12} and folate are derived from microbial sources [29 30]. Pregnant mothers are widely supplemented with folate in developed countries to avoid facial clefting, neural tube and cardiac defects. Folate and B_{12} are relevant as the catalysts for methylation reactions in epigenetic marking – the relevance of this to immune system development will be further developed below.

Given that intestinal motility is reduced in pregnancy through the secretion of progestogens, one cannot safely extrapolate physiological studies in non-pregnant individuals: nevertheless, it is likely that lower small intestinal microbial biomass increases in pregnancy and probably also the microbial-host molecular exchange of nutrients.

Development of the maternofetal interface

The basis of maternofetal contact and the organ for molecular exchange is the placenta. The timelines of the development of this interface and of the different phases of embryogenesis will be considered in terms of maternofetal molecular transfer and fetal immune development.

Both mice and humans have a hemochorial placenta **[G]**, because during the process of decidualization the fetal trophoblast invades both the uterine epithelium and the endothelium of the maternal vessels, so the syncytial cellular outer trophoblast layer is directly bathed with maternal blood (**Figure 2A**). The outer trophoblast structure has developed from the trophoectoderm becoming vascularized via the mesoderm of the allantois **[G]**. The allantois emerges from the posterior end of the embryo forming a vascular labyrinth as the trophoblast invades the mouse maternal decidua at embryonic day 12.5 (E12.5) (**Figure 2A**) [31].

As the placenta is formed, the embryonic immune system is in a phase of stem cell formation in the splanchnopleuric mesenchyme surrounding the developing heart, the aortogonadomesonephros (AGM). In the mouse at around E10.5 the phase of tissue migration and lineage progenitor expansion starts with lymphopoiesis in the fetal liver and the thymus (**Figure 2B**). This is aligned with the vascularization of the trophoblast and decidualization, yet effects of maternally derived molecules on the developing fetus can occur even without this intimate contact when present in sufficiently high concentration, as

evidenced by the teratogenic effects of a short-lived high dose of alcohol at E7 in the mouse [32]. Nevertheless, pharmacological studies of placental drug transfer indicate greater fetal exposure to a standardized dose at later points in gestation [33]. This is inextricably linked to the fetal demands for oxygen and nutrients for growth: as the trophoblast area expands and the intrahemal distance is reduced, measurements with radioactive compounds have shown that both facilitated and diffusive exchange of metabolites become increased between E16 and E19 [34].

The relationship between the fetus and the mother cannot be entirely mutualistic. There must be resource transfer from mother to the fetus for it to develop. Even in times of famine, precious resources diverted from the mother to the fetus may also be consumed by the trophoblast. This equilibrium is highly regulated through epigenetic imprinting, which co-evolved with placentation. This results in placental development and increased size being driven by paternally expressed alleles in the fetus (e.g. insulin-like growth factor (IGF 2)), and counterbalanced by genes expressed from maternal alleles (e.g. IGF type 2 receptor that targets the protein for degradation) [35]. Remarkably, most sex-specific epigenetic imprinting affects genes expressed in the placenta or the brain: imprinting therefore not only determines resource allocation between fetus and mother but also maternal endocrine responses and behaviour necessary to successfully nurse the offspring [36]. The potential susceptibility of imprinting to external influences in humans was shown in a remarkable study on babies born from pregnancies in Holland in the famine of the 1944-1945 winter. Even 6 decades later, individuals born during the famine showed less DNA methylation at the *IGF2* locus, possibly reflecting their demand for nutrition in fetal life, compared with unexposed siblings of the same gender [37] (also see BOX 1).

Although genomic imprinting is not known to have major direct effects on immune system development in vertebrates [38] it has major indirect effects through regulating the placental interface for passage of maternal antibodies. Such antibodies transmit maternal immune memory of pathogens [11] and non-pathogenic members of the microbiota to the neonate [39]. The timing of antibody transmission from mother to offspring is variable between different species, but in general it is a transgenerational effect that is very sensitive to adequate nutrition because the physiological expense of the immune response competes with nutrients for maternal metabolism and fetal growth [40]. Maternal antibodies can be taken up into the circulation and tissues of the offspring to transfer immune memory, which may neutralize pathogens or components of the microbiota [11]. These antibodies are capable of limiting infectious disease during the period of neonatal immune immaturity and can shape the development of endogenous antibody repertoires in the offspring during early life [12].

Exposure of the infant to the maternal microbiota (see also BOX 2)

Direct in utero exposure to live organisms of the maternal microbiota.

Although the biomasses of the host and its microbiota are rather well separated, this is not absolute, and very small numbers of live microbial organisms can be detected in systemic organ systems of the host both in animal models and in humans [41 42]. During late pregnancy and in the immediate postnatal period, translocation of intestinal and oral microbes is increased in both experimental rodents and in humans [43-45]. This results in microbes being present in the placenta and in the milk [43,46]. At least for placental and fetal tissues, microbial numbers must be maintained at extremely low levels, otherwise the pre-term birth or stillbirth complications of intrauterine infection will ensue [47]. Nevertheless, there is evidence

that these organisms contribute to the early colonization of the postnatal infant [43]: they may also play a role in supporting the developing fetal immune system in utero, although the extent of this is uncertain.

Exposure of the fetus and neonate to penetrant maternal microbial molecules.

In experimental mice one can show that the development of the immune system in the fetus is driven by maternal microbial molecules independently of actual penetration of live microbes into maternal or conceptus tissues. For the most part, the effects of the microbiota on immune system development (as well as their effects on other organ systems) have been determined by comparing colonized and germ-free animals [48].

As germ-free animals are born to a germ-free dam, and colonized animals are born to a colonized dam, comparing these two hygiene statuses does not provide information on the effects of the maternal microbiota on the development of the immune system of her pups. However, the effect of the maternal microbiota alone can be addressed by treating germ-free pregnant female mice with live *Escherichia coli* that lacks the synthetic pathways for the essential bacterial amino acids, D-alanine and diaminopimelic acid [49]. This strain can be grown in culture by providing these amino acids as supplements, but it does not permanently colonise the mouse intestine as D-alanine and diaminopimelic acid are not synthesized or available in the host to support bacterial replication in the intestinal luminal environment. The big advantage of this system is that the pregnant dam returns to germ-free status before delivery of her pups, and so the pups themselves are germ-free. Thus, one can compare the immune responses in the pups born to the transiently colonized mothers with pups born to a control germ-free mother (**Figure 3**).

The results show that, compared with control germ-free pups, germ-free pups born to transiently colonized dams harbour higher numbers of class 3 innate lymphoid cells (ILC3s) in the small intestine and have increased numbers of CD11c+F4/80+ intestinal mononuclear cells in both the small and large intestine [50]. These effects are durable from approximately postnatal day 5 until at least day 60. The effects are not limited to the leukocyte populations as intestinal mucosal transcriptional signatures (predominantly from epithelial cells) are profoundly reshaped in pups born to gestationally colonized dams. In particular, intestinal tissues from pups show increased expression of antibacterial peptides (including C-type lectins of the Reg family and defensins), increased cell division and differentiation of epithelial cells, higher expression of mucus and ion channels, increased metabolism of dietary xenobiotics, bile acids and complex lipids, changes in sugar metabolism and effects on antibody responses (including increased expression of the polymeric immunoglobulin receptor that is responsible for transporting IgA through the epithelial layer into the intestinal lumen). Strain combination experiments showed that many of these effects are dependent on the transiently colonized mother expressing antibodies, implying that antibody transfer to the fetus and neonate enhances the gestational colonization effect [50].

As the transiently colonizing *E. coli* strain is unable to replicate in vivo, it is possible to carry out isotope flux experiments using metabolically labelled bacteria. These experiments showed that there is substantial transfer of metabolites from the intestinal bacteria into the mother, across the placenta and into the fetus **[50]**. Because there is a long dwell time of bacterial metabolites that are transferred into maternal tissues, the uptake of bacterial metabolites continues through to the milk. There are likely to be multiple different molecules that are driving the process, but one group that is transferred without secondary metabolism

comprises ligands for the aryl hydrocarbon receptor (AhR). Administration of an authentic AhR ligand (indole-3-carbinol) to pregnant female mice showed that this was sufficient to explain the effect of maternal intestinal bacteria on fetal ILC3 populations[50].

Such gestational colonization effects are likely to be important in preparing the neonatal mammal for its own microbial colonization. Experiments showed that gestational colonization effects prepared the pups to have a more intact intestinal barrier and blunted the responsiveness of their splenocytes to systemic challenge with lipopolysaccharide [50].Clearly, such experiments would be unethical and technically unfeasible in humans, but they provide insight into possible ways in which the maternal microbiota may shape the fetal immune system in humans. Although mice continue to take up IgG from the maternal milk through the neonatal intestine until about postnatal day 12 [51], human IgG uptake is essentially complete via placental transfer at birth. However, most human transplacental IgG uptake occurs in the last month of pregnancy [52]. One may speculate that the immaturity of intestinal function that is seen in babies born pre-term (for example, their increased susceptibility to inflammatory necrotizing enterocolitis as a result of enteral feeding [53] and the relative protection afforded by feeding pre-term infants human colostrum [54],which does contain IgG) can be explained, at least partly, by altered exposure of the neonate to maternal microbial components.

Postnatal lactation and the neonatal microbiota

Earlier in this Review, we described the transgenerational transfer of immune memory from mother to offspring that results from the transmission of systemic antibodies (mainly IgG) that can limit systemic infections. Whereas this is largely transplacental and antenatal in humans, in some species such as ungulates this happens postnatally through the colostrum. Mice are in an intermediate position with both pre-natal and postnatal phases of maternal antibody transfer.

In addition to containing antibodies specific for potential pathogens, milk contains antibodies induced by the maternal microbiota. These antibodies mainly comprise secretory IgA (SIgA) and SIgM, but also include IgG isotypes in mice, and they can protect the immature mucosal surfaces of the offspring. The evidence for this originates from heterozygous breeding studies using scid/+ mice. When these mice were born to and nursed by *scid/scid* dams (mated with wild-type males) the heterozygous offspring induced endogenous mucosal SIgA early, at approximately day 15, in the face of no antibody content in the maternal milk. In the converse experiment, where *scid/+* pups are born to and nursed by wild-type dams (mated with *scid/scid* sires) endogenous mucosal SIgA induction was delayed until after weaning [55]. This work was later developed in the $J_H^{-/-}$ strain that is selectively deficient for antibodies to confirm that; one, the milk antibody protected the offspring against the intestinal microbiota; two, that this was a mucosal protection effect, because parenteral replacement of the early phase of IgG did not affect early induction of endogenous IgA when the maternal milk lacked antibodies; and three, that the maternal milk antibodies prevented microbes from translocating to the mesenteric lymph nodes in the pups [56] (**Figure 5A**).

This approach has been taken further still using crosses from the pIgR-deficient strain, where $pIgR^{-/-}$ males were crossed with $pIgR^{+/-}$ females or $pIgR^{+/-}$ males were crossed with $pIgR^{-/-}$ females. In both cases, heterozygous and homozygous pups were obtained from each cross (**Figure 5B**). In the cases where the dam was pIgR-deficient there was no IgA or IgM antibody secretion into the milk. Whether or not the pup was able to secrete endogenous

mucosal antibodies the maternal antibody effect both protected the pup against an inflammatory transcriptional signature of the intestinal mucosa and differentially shaped the composition of the resultant endogenous microbiota, especially reducing the long term (day 70) proportions of Proteobacteria present [57]. In mice IgG is transferred from the mother to her offspring via the neonatal (FcN) receptor both across the placenta and via the neonatal duodenum. Using a heterozygous breeding strategy for FcN [$FcN^{-/-}$ males crossed with $FcN^{+/-}$ females] supported by germ-free experiments and experiments with colonised mice deficient for all antibody isotypes or selectively for IgA, it was shown that the postnatal uptake of maternal T-independent IgG2b and IgG3 antibodies limits the development of neonatal follicular helper T (T_{FH}) cell and germinal center responses against intestinal microbes[39].

Therefore, although there are antimicrobial effects of other antimicrobial components of milk (including lactalbumin, lysozyme, lactoferrin and lactoperoxidase), secreted milk antibodies are protective for the neonatal intestinal mucosal surface, limiting penetration of the incoming intestinal microbes into systemic tissues and limiting B cell and inflammatory responses in the neonatal mucosa itself. This results in a less inflammatory composition of the microbial consortia that stably colonized the early life mouse intestine. This protective effect of secreted milk antibodies is independent of transgenerational Ig uptake in the neonatal intestine. Establishment of a healthy microbiota – which is likely coupled to development of the early life intestine – in this early phase of life is extremely important. In less developed countries, severe protein-energy malnutrition characterised by dysbiosis, small intestinal enteropathy and growth stunting can supervene at the time of weaning [58,59]. A mammalian mother is preparing her offspring through the direct effects of nutrition and passage of immune memory, as well as the indirect effects of antibody-enhanced molecular exchange to stablise the earliest stages of host-microbial mutualism.

Epilogue

In this Review we have described how the intestinal microbiota and nutritional status of the mother interact in a number of distinct ways during pregnancy and the early life of the offspring. In addition to enhancing the all-important energy yield, the maternal microbiota assists with micronutrient provision and xenobiotic metabolism. There is rather pervasive penetration of maternal microbial compounds, which can reach the fetus via the placenta and the milk, enhanced by maternal antibody transfer. Uncoupling defined exposure of pregnant mice to live microbes from permanent colonization shows that there is a large spectrum of immune and non-immune effects of maternal microbial molecular exposure: the durability of these effects and the established effects of known environmental xenobiotics suggests that there are likely to be underlying epigenetic alterations.

Both gestational colonization driven reprogramming of the neonate, as well as direct secretory antibody protection, prepare the immunologically immature intestinal mucosa for its own tsunami of colonization by endogenous intestinal microbes and help ensure that healthy consortia are formed which will protect the offspring from diseases throughout life.

Figure legends:

Figure 1: Maternal stress weakens maternal and offspring immunity. Maternal stress, such as the exposure to lipopolysaccharide or malnutrition, induce the release of adenocorticotropin (ACTH) from the pituitarity gland. ACTH triggers the release of corticosteroids from the adrenal glands leading to suppression of the maternal immune system. Besides a generally weakened response of the pregnant women to infections, the lower rate of immunoglobulin production in the bone marrow results in less efficient transfer of immunoglobulins to the offspring via placenta and milk.

Figure 2: Placenta development and fetal haematopoiesis A. Schematic view of murine placental development. Key points at embryonic day (E) 3.5, 6.0, E 8.5, E 12.5 and E18 are shown. Placenta development starts upon implantation of the blastocyst into the maternal endometrium. The outer layer of the blastocyst becomes the trophoblast, which later forms the outer layer of the placenta. The maternal endometrium contributes the maternal decidua of the placenta, which is accommodated by maternal spiral arteries guarantying the blood supply of the placenta. Fetal umbilical arteries form a labyrinth while invading the placenta to form an arterio-capillary-venous system. B. Time line of murine and human haematopoiesis during embryonic and fetal development.

Figure 3: Model of transient gestational colonization of germ-free mice. Time-pregnant germ-free mice were gavaged with 10^{10} c.f.u. *E. coli* HA107 between E8 and E16 or kept germ-free throughout pregnancy. *E. coli* HA107 is an auxotrophic strain harbouring mutations in the synthesis pathway of meso-diaminopimelic acid and of D-alanine. It can thus only survive in supplemented culture and colonizes a germ-free murine intestine transiently for 24-72 hours. The pregnant dams returned to germ-free status before delivering their germ-free pups, which can subsequently be analysed for the effect of maternal gestational colonization on offspring immunity and disease susceptibility.

Figure 4: Epigenetic re-programming during mammalian development. Key developmental steps are shown in relation to epigenetic modifications and gene expression patterns. Early after fertilization of the oocyte, DNA methylation is erased, enabling the expression of pluripotency-associated genes. Developmental genes required for the differentiation of specific cell types are still repressed by histone H3K27 methylation. As development proceeds, pluripotency-associated genes and paternal or maternal imprinted genes need to be silenced permanently by DNA methylation. At the same time, an increase in H3K4 methylation and a reduction in H3K27 methylation enable the expression of developmental genes.

Figure 5: Murine experimental models of maternal antibody deficiencies. A. The effect of maternal antibodies on the offspring can be studied by using antibody-deficient mice ($J_H^{-/-}$). $J_H^{+/+}$ males are crossed with $J_H^{-/-}$ females to obtain antibody-sufficient ($J_H^{+/-}$) pups that are nursed by antibody-deficient ($J_H^{-/-}$) dams. As a control, $J_H^{-/-}$ males are crossed with $J_H^{+/+}$ females to obtain antibody-sufficient ($J_H^{+/-}$) pups that are nursed by antibody-sufficient ($J_H^{+/+}$) dams. B. To distinguish between the roles of IgG antibodies that are passively transferred

from the mother to the offspring and of actively transferred maternal SIgA that are secreted into the maternal milk via the polymeric Ig receptor (pIgR), $pIgR^{+/-}$ males are crossed either with $pIgR^{-/-}$ (pIgR-deficient) or $pIgR^{+/+}$ (pIgR-sufficient) females. The offspring of these matings always contain pIgR-deficient ($pIgR^{-/-}$) and pIgR-sufficient ($pIgR^{+/-}$) pups that can again be compared in regard to the role of endogenous antibody secretion.

BOX 1: EPIGENETIC ENVIRONMENTAL INFLUENCES ON THE DEVELOPING FETUS.

As illustrated for imprinting, epigenetics has a profound effect on the developing fetus. The maternal and paternal marks survive the genome-wide demethylation that occurs in embryonic somatic cells at the blastocyst stage. This leaves imprint control centres that are read into appropriate differential gene expression in the developing embryo (**Figure 4**). It also leaves virgin genetic territory on which the epigenetic controls can be established to determine the proliferative and differentiation fate of pluripotent cells. In contrast, in primordial germ-line cells the parental imprints are erased and re-established in a gender-specific fashion during gamete development. The epigenetic processes are subject to external influences. The classical example of this is the brown Agouti (A/A) mouse, where coat colour of A^{vy}/a pups born to an a/a (black) mother can be determined by methylation status of the retrotransposon regulatory region in the A^{vy} gene. This methylation can be manipulated by supplementation with vitamins B12 and folate [60]; as described above, de novo synthesis by members of the microbiota contributes to body stores of these dietary micronutrients even without coprophagia. Other known epigenetic modifiers are cigarette smoke (which in addition to other effects results in hypomethylation of the aryl hydrocarbon repressor gene [61]) and alcohol (which in addition to critical effects on CNS development also causes deficient lung development and defects in pulmonary innate immunity [62]).

The developing immune system requires phases of progenitor cell expansion and tissue migration and later lineage establishment and maturation. Epigenetic controls that are established through the different processes of methylation, histone modification and untranslated RNA expression are best described for lymphocyte lineage differentiation of different T cell subsets in the adult animal [63]. The processes that guide differentiation of hemapoietic stem cells that characterize the lineage progenitors are emerging [64-66]. It is reasonable to assume that the pervasive maternal gestational effect from the microbiota will also have an epigenetic basis because of its durability and the environmental precedents cited above. The details of these mechanisms are still under investigation.

BOX 2: MATERNAL MICROBIOTA EFFECTS.

Live microbial exposure of the trophoblast/placenta: Although the placenta is generally believed to be a sterile body organ, recent literature points towards a possible low-grade colonization of placental tissue with commensal bacteria. Colonization was however estimated to be low and sampling of human placenta without contamination is still a challenge that may have influenced the results.

Endobiotic microbial molecular transfer: Metabolites or fragments of the maternal endogenous microbiota can reach the maternal serum and systemic sites and can thus be transferred to the unborn fetus via the placenta or to the newborn child through maternal milk.

Nutrition: Dietary components in the maternal intestine can be further metabolized by

members of the intestinal microbiota and the resulting products can be absorbed by the maternal organism and subsequently transferred to the offspring. In addition, the maternal diet can influence composition or transcriptional state of the maternal intestinal microbiota itself.

Xenobiotics: As with dietary components, several members of the human intestinal microbiota have the potential to metabolize xenobiotic chemicals originating from plant or pharmaceutical sources in the maternal gut and thereby alter the chemical exposure of the fetus.

BOX 3: *E. COLI* AS MODEL ORGANISM FOR GESTATIONAL COLONIZATION.

The effects of gestational colonization on the development of the neonatal immune system have only been performed by using the so far uniquely available reversibly colonizing commensal bacterium, *E. coli* HA107. Although *E. coli* is only a rare member of the murine intestinal microbiota, it is present within the human intestine, especially at early life [67,68]. When we analyzed pups born to fully colonized SPF mice or mice colonized with the Altered Schedler Flora and compared them to the offspring born to gestationally *E. coli* colonized mothers, we observed the same alterations on the immune system of the offspring in comparison to pups born to untreated germ-free dams. We are currently working on generating other auxotrophic commensal bacterial strains that can be used for transient gestational colonization to corroborate our results.

Glossary:

Thalidomide: A drug primarily prescribed as a sedative or hypnotic. It was used against nausea and to cure morning sickness in pregnant women until it was discovered that it caused absence of limbs of the fetus at birth.

*Hypothalamo-pituitary-adrenal (HPA) axi*s: One of the major neuroendocrine systems that controls reactions to stress, the immune system, digestion, emotions etc. It consist of a complex set of feed-forward and feedback mechanisms between the hypothalamus, the pituitary gland and the adrenal cortex. Neuroendocrine neurons in the hypothalamus produce corticotropin-releasing hormone, which acts on the anterior pituitary gland that subsequently produces adrenocorticotropic hormone (ACTH). ACTH induces the adrenal gland to release glucocorticoids, such as cortisol.

Coprophagia: Eating of feces, which is normal behavior for many animals.

Hemochorial placenta: The type of placenta present in humans and some rodents, where maternal blood is in direct contact with the chorion.

Allantois: A bag-like structure that forms part of the developing conceptus, which helps the embryo in nutrition and excretion.

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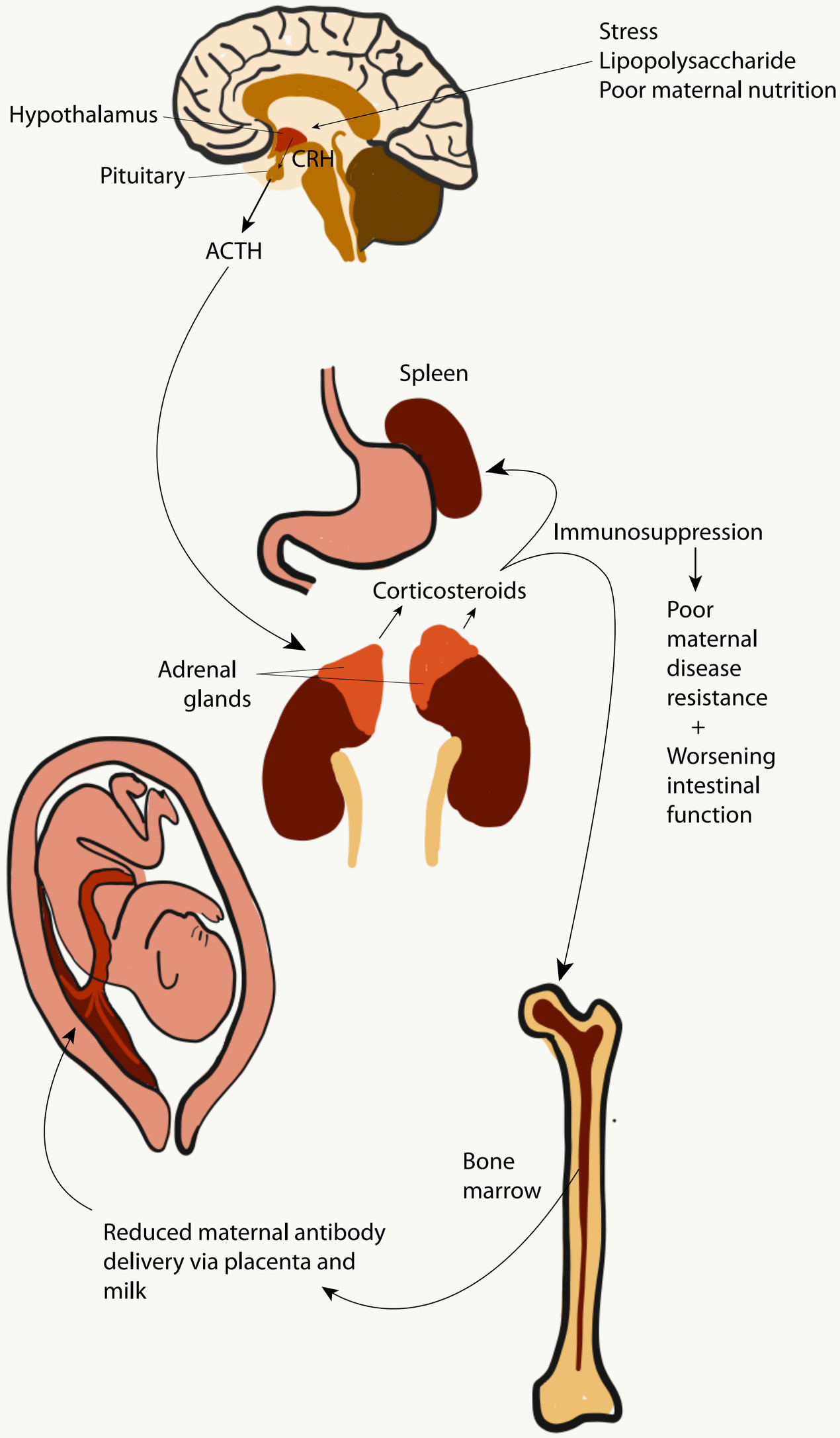
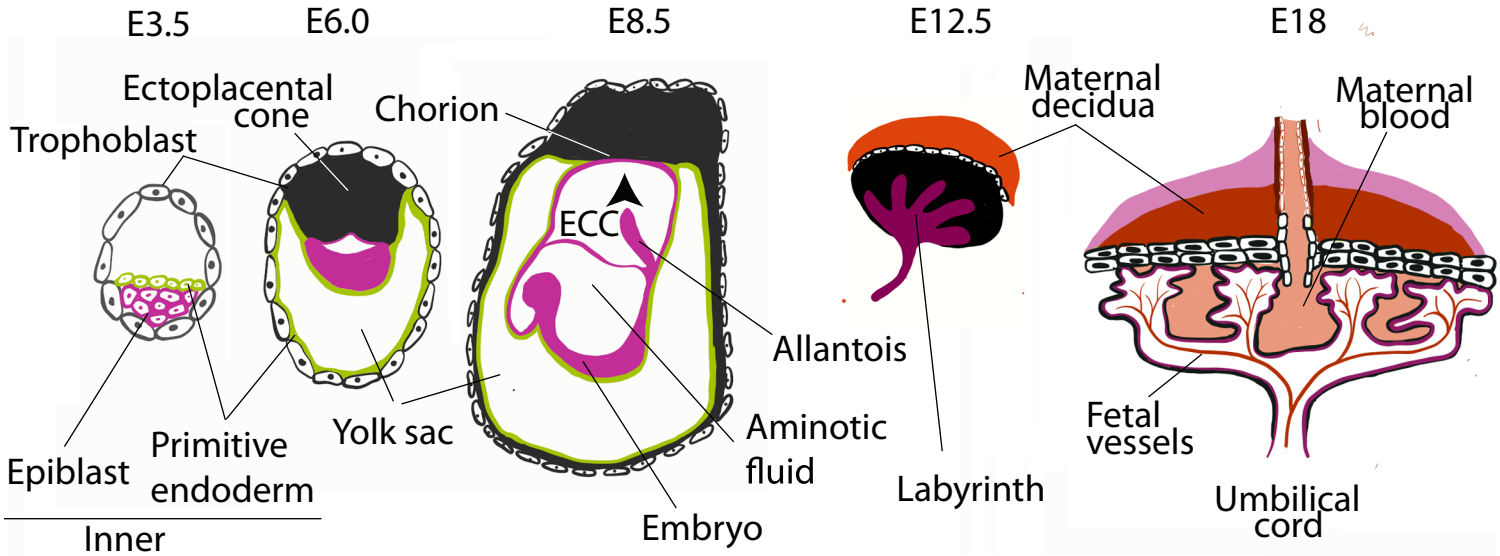
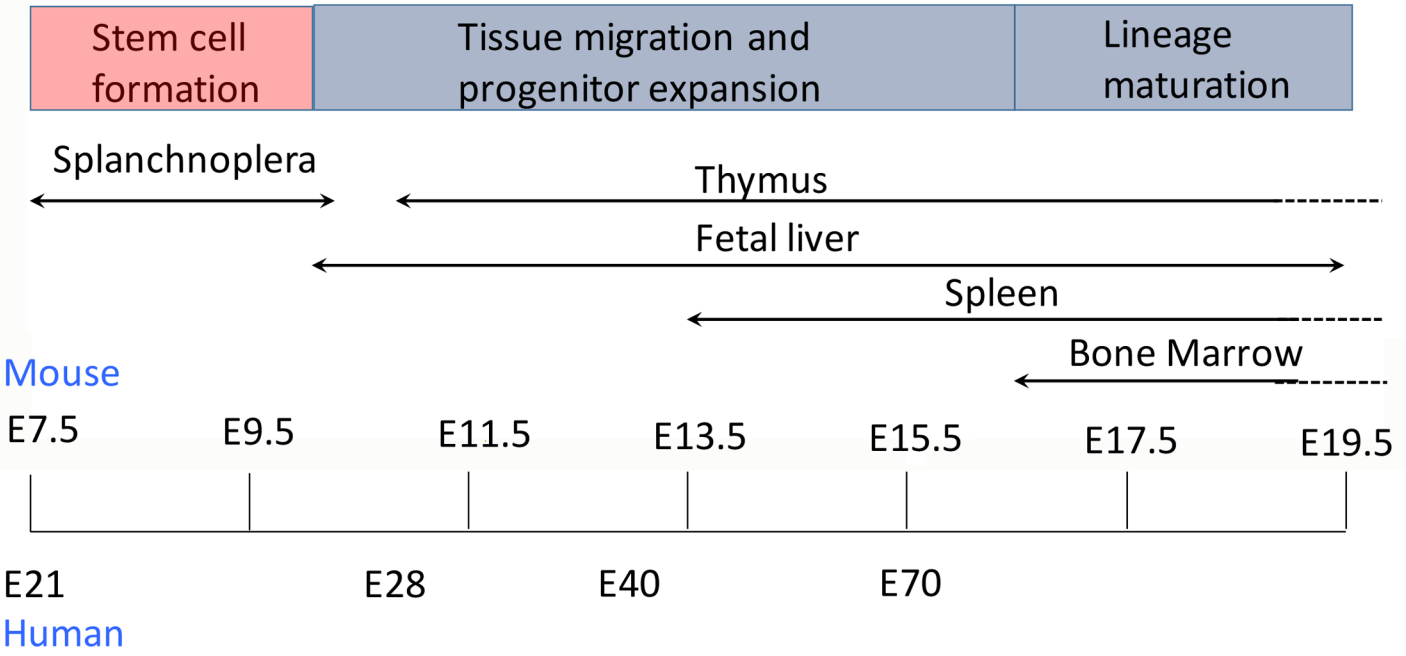
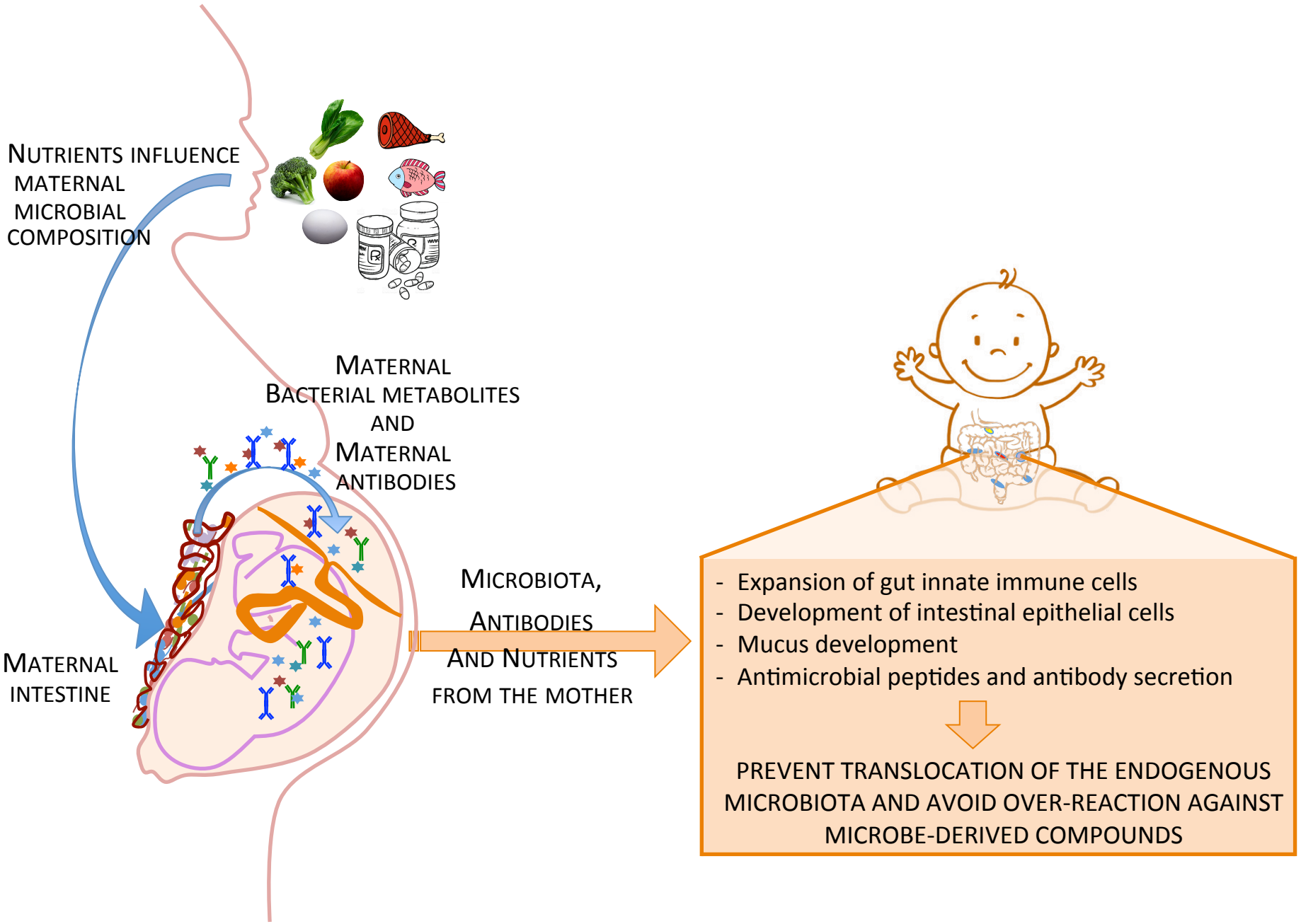

Stress
Lipopolysaccharide
Poor maternal nutrition
Hypothalamus
CRH
Pituitary
ACTH
Spleen
Immunosuppression
Corticosteroids
Adrenal glands
Poor maternal disease resistance + Worsening intestinal function
Bone marrow
Reduced maternal antibody delivery via placenta and milk

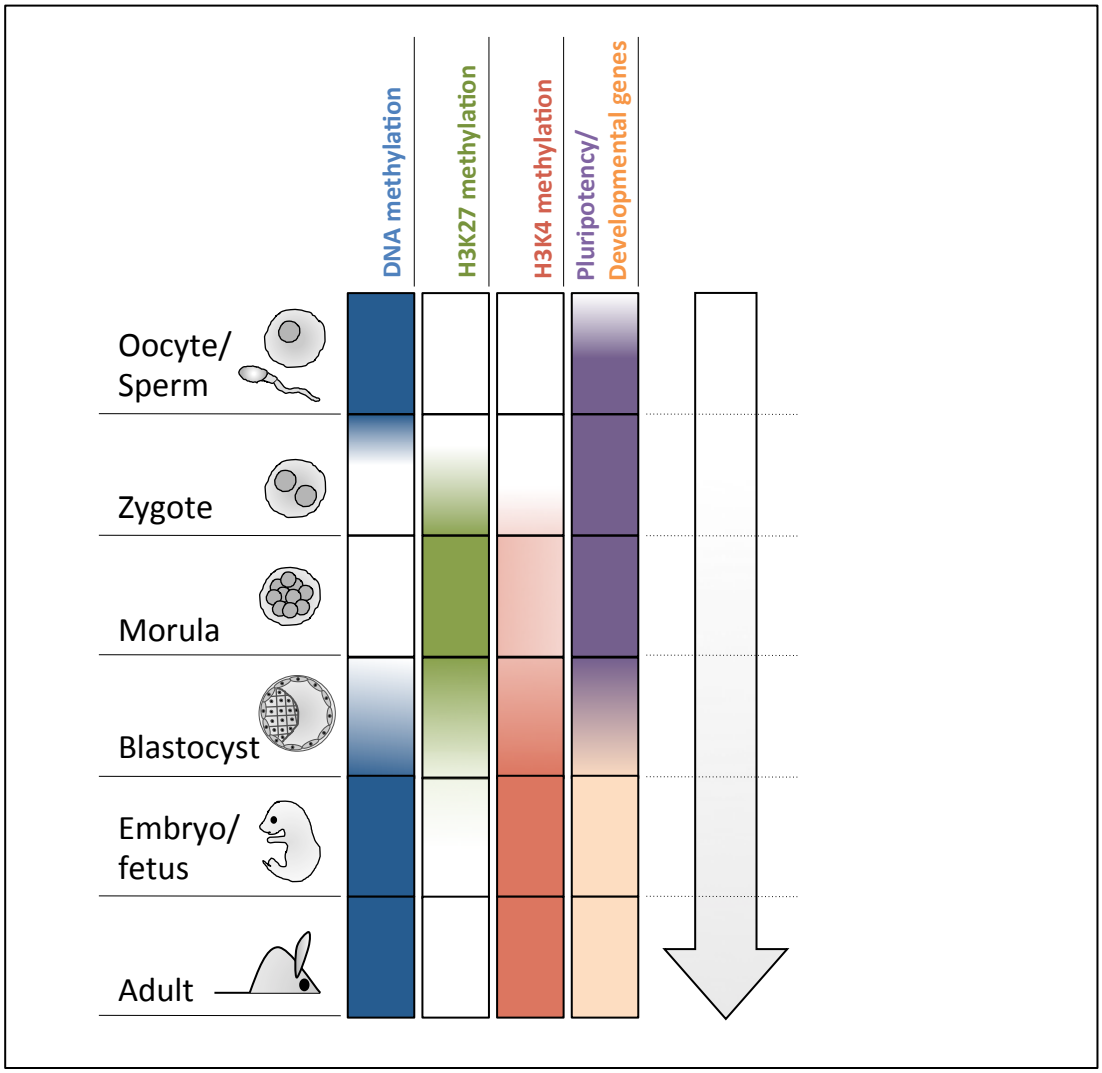
A)


E3.5
E6.0
E8.5
E12.5
E18
Ectoplacental cone
Trophoblast
Chorion
ECC
Allantois
Maternal decidua
Maternal blood
Yolk sac
Aminotic fluid
Fetal vessels
Epiblast
Primitive endoderm
Embryo
Labyrinth
Umbilical cord
Inner cell mass

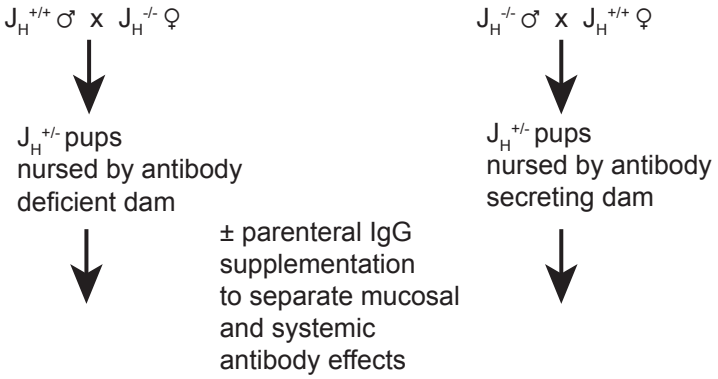
B)


Stem cell formation
Tissue migration and progenitor expansion
Lineage maturation
Splanchnoplera
Thymus
Fetal liver
Spleen
Bone Marrow
Mouse
E7.5
E9.5
E11.5
E13.5
E15.5
E17.5
E19.5
E21
E28
E40
E70
Human

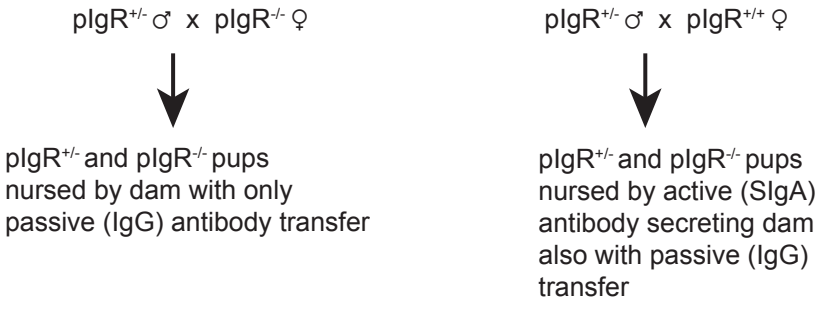

NUTRIENTS INFLUENCE MATERNAL MICROBIAL COMPOSITION
MATERNAL BACTERIAL METABOLITES AND MATERNAL ANTIBODIES
MATERNAL INTESTINE
MICROBIOTA, ANTIBODIES AND NUTRIENTS FROM THE MOTHER
- Expansion of gut innate immune cells
- Development of intestinal epithelial cells
- Mucus development
- Antimicrobial peptides and antibody secretion
PREVENT TRANSLOCATION OF THE ENDOGENOUS MICROBIOTA AND AVOID OVER-REACTION AGAINST MICROBE-DERIVED COMPOUNDS

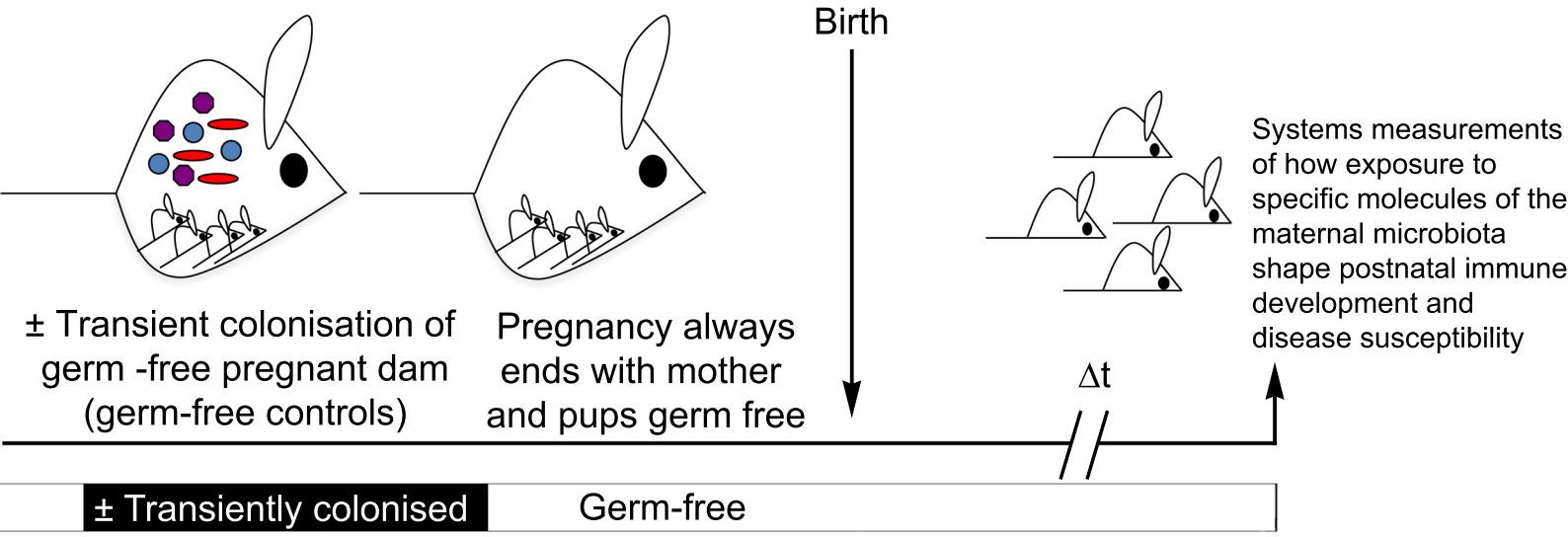

DNA methylation
H3K27 methylation
H3K4 methylation
Pluripotency/
Developmental genes
Oocyte/
Sperm
Zygote
Morula
Blastocyst
Embryo/
fetus
Adult

A) Effects of maternal antibody deficiency



B) Effects of passive (IgG) or active (mucosal SIgA) maternal antibodies




Birth
± Transient colonisation of germ -free pregnant dam (germ-free controls)
Pregnancy always ends with mother and pups germ free
Δt
Systems measurements of how exposure to specific molecules of the maternal microbiota shape postnatal immune development and disease susceptibility
± Transiently colonised
Germ-free