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SPOTLIGHT

Gene-environment interactions: aligning birth defects research with complex etiology

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ABSTRACT

Developmental biologists rely on genetics-based approaches to understand the origins of congenital abnormalities. Recent advancements in genomics have made it easier than ever to investigate the relationship between genes and disease. However, nonsyndromic birth defects often exhibit non-Mendelian inheritance, incomplete penetrance or variable expressivity. The discordance between genotype and phenotype indicates that extrinsic factors frequently impact the severity of genetic disorders and vice versa. Overlooking gene-environment interactions in birth defect etiology limits our ability to identify and eliminate avoidable risks. We present mouse models of sonic hedgehog signaling and craniofacial malformations to illustrate both the importance of and current challenges in resolving gene-environment interactions in birth defects. We then prescribe approaches for overcoming these challenges, including use of genetically tractable and environmentally responsive *in vitro* systems. Combining emerging technologies with molecular genetics and traditional animal models promises to advance our understanding of birth defect etiology and improve the identification and protection of vulnerable populations.

KEY WORDS: Gene-environment interaction, Birth defect, Orofacial clefts, Holoprosencephaly, Organoid, Microphysiological model, Sonic hedgehog

Introduction

A multifactorial model of birth defect etiology can be traced back to F. Clarke Fraser's research in the 1950s. At the time, the recent discovery that the uterus was not impervious to the environment led many developmental biologists to pursue the emerging study of mammalian teratogens and teratology at the expense of genetics. Fraser, a geneticist by training, retrospectively described it as a period during which 'the pendulum of opinion was swinging away from the idea that malformations are genetic in origin…to the other extreme – that malformations are mostly caused by environmental factors' (Fraser, 2008). Following this change in momentum, birth defects research diverged. Josef Warkany advanced the nascent field of teratology, while medical genetics found a foothold and flourished under the leadership of Victor McKusick. Fraser, convinced of the importance of both genetic and environmental influences, instead sought to integrate these two approaches for explaining abnormal development.

Having been provided cortisone to investigate its potential to disrupt neural tube development in mice, Fraser unexpectedly discovered that maternal treatment induced cleft palate in the pups (Fraser, 2008). On a hunch, Fraser administered cortisone to each of the mouse strains available to him. Cleft palate was inducible across five strains, but Fraser noted that incidence was strain, and therefore genotype, dependent (Fraser and Fainstat, 1951). This early experiment in teratogenetics, Fraser's term for the study of gene-environment interaction in developmental disorders, illustrated the importance of combinatory insults and risk interactions. Even so, medical genetics and teratology largely progressed independently, each field working on the same puzzle with a different set of pieces.

Although it is true that traditional epidemiological and genetic approaches have resolved the causes of birth defects that are strongly genetic or environmental in nature, it is now widely recognized that complex interactions between genetic and environmental influences shape the nature and severity of most birth defects. In spite of this recognition, it is our opinion that the advent of modern genomics in recent decades has led to another sea change, one in which a genetics-forward approach dominates the study of abnormal development. We believe, as Fraser did, that there is more to the story.

Gene-environment interactions (also abbreviated to GxE) occur when genetic and environmental influences additively or synergistically contribute to a phenotypic effect. Environment, in this context, may broadly refer to any influence that is not genetic in nature, including toxin and toxicant exposure, maternal infection, hypoxia, and macromolecule or micronutrient excesses or deficiencies. A widely recognized example of a gene-environment interaction is phenylketonuria, an autosomal recessive disease caused by mutation in phenylalanine hydroxylase, which is exacerbated as phenylalanine intake exceeds an affected individual's ability to metabolize it. In practice, individuals lacking a functional copy of phenylalanine hydroxylase exhibit phenotypes including intellectual disability, seizures and behavioral problems, the severity of which correlates with phenylalanine intake, whereas a single intact allele is considered protective against phenylketonuria. Although illustrative, this simple type of gene-environment interaction – a homozygous genetic aberration acting in concert with an otherwise innocuous environmental influence – is not universally representative of the phenomenon. Rather, interactions can take several forms (described by Khoury et al., 1988) in which environmental or genetic influences drive a phenotype that is modified by additional factors. In fetal alcohol spectrum disorder, for instance, prenatal alcohol (ethanol) exposure is sufficient to disrupt development of the brain and face, although certain gene variants appear to exacerbate these outcomes (reviewed by Lovely et al., 2017). Thus, the impact of the primary insult, maternal alcohol exposure, is modified by genotype. Importantly, the phenotypic variability seen in fetal alcohol spectrum disorders highlights the continuous nature of many multifactorial diseases, including some of the most common human structural birth defects.

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Craniofacial birth defects: a face of gene-environment outcomes

Craniofacial birth defects, such as those seen in fetal alcohol spectrum disorders, illustrate both the challenges and opportunities of studying gene-environment interactions. The head and face develop through precisely coordinated expansion and fusion of embryonic growth centers comprising multiple migrating and differentiating cell populations. Consequently, congenital craniofacial abnormalities are relatively common, and the functional and societal importance of the face makes these malformations particularly impactful for patients and their families.

Orofacial clefts (OFCs) of the lip and palate are the most prevalent human craniofacial birth defects and affect approximately 1 in 700 newborns (Leslie and Marazita, 2013). Most OFCs occur in apparent isolation of other malformations and are considered nonsyndromic. The vast majority of these cases do not follow Mendelian inheritance patterns, although genetics undeniably plays a substantial role in modifying risk. For example, OFC incidence varies by background and demonstrates at least some familial heritability (Watkins et al., 2014). Accordingly, OFCs have long been thought to arise from gene-environment interactions (reviewed by Dixon et al., 2011; Krauss and Hong, 2016; Lovely et al., 2017), although efforts to understand this complex etiology have largely focused on the genetic side of the equation. Dozens of OFC risk loci have been identified by employing complementary genetic approaches, including genome-wide association studies (reviewed by Beaty et al., 2016; Leslie and Marazita, 2013; see also OMIM 119530). Similarly, despite frequent postulation of environmental contributions to clefting, no exogenous factor is known to exhibit strong penetrance, and commonly implicated factors either only slightly increase risk (e.g. maternal exposure to cigarette smoke) (Hackshaw et al., 2011) or have shown mixed results in epidemiological studies (e.g. maternal alcohol exposure) (Bell et al., 2014). In most cases, the functional consequence of identified genetic variants and environmental influences, and how these factors may interact to produce OFCs, remains unknown.

In considering gene-environment interactions in craniofacial malformations, a useful counterpart to OFCs is the related malformation holoprosencephaly (HPE). Defined by deficient development of the median forebrain, HPE frequently co-occurs with facial abnormalities including OFCs. At its most severe, HPE results in cyclopia, characterized by a single central eye. Although relatively rare in newborns, HPE has an estimated prevalence of 1 in 250 conceptuses (Petryk et al., 2015), suggesting that it is among the most common human embryonic malformations. Furthermore, although chromosomal abnormalities account for approximately 1 in 3 HPE cases (Petryk et al., 2015), the remaining cases are considered etiologically heterogeneous with genetics playing an important, but apparently incomplete, role. For example, of the 17 (and counting) genes associated with HPE, mutations in the four most common are detected in only 25% of cases (Roessler et al., 2018; Tekendo-Ngongang et al., 2000). Even in this subset of gene-associated cases, causative mutations are almost exclusively heterozygous and considered to act as autosomal dominant but with incomplete penetrance and highly variable expressivity. Increasingly, rare gene variants associated with HPE are being identified (Hughes et al., 2020; Kruszka et al., 2019a,b), which may contribute to the phenotypic variability of this condition and increase the number of potential gene-gene interactions, although documented instances of 'multiple-hit' mutation events are exceedingly rare in HPE (Roessler et al., 2018). As with OFCs, gene-environment interactions are considered central to HPE etiology, with the identification of specific interactions remaining limited and prevention strategies largely unavailable. However, findings spanning decades of research across multiple fields have coalesced to support a model of gene-environment interactions in HPE etiology.

Sonic hedgehog signaling and holoprosencephaly: a lens to view gene and environment

Just 4 years after the historic elucidation of DNA's double helix, ranchers in the western United States observed sheep born with craniofacial malformations including cyclopia, the hallmark phenotype of severe HPE, and alerted the United States Department of Agriculture (USDA). In a now science-famous story, USDA researchers traced these birth defects to maternal grazing on the plant *Veratrum californicum* and a teratogenic alkaloid that they dubbed cyclopamine (Chen, 2016; Keeler, 1978). Without the tools to probe the mechanism of cyclopamine-mediated birth defects, this curiosity of teratology faded into the background. Meanwhile, the modern genetics era dawned. Nobel prize-winning fruit fly studies identified genes now known to be central mediators of development and disease, including one named *hedgehog*. Within two decades, a knockout mouse for sonic hedgehog (*Shh*), a mammalian *hedgehog* homolog, was generated and found to have severe craniofacial malformations, including cyclopia (Chiang et al., 1996). Astute developmental biologists reconsidered those one-eyed sheep and subsequently demonstrated in chicks, mice and mammalian cells that cyclopamine-induced birth defects result from the inhibition of the Shh signaling pathway component smoothened during embryonic development (Chen et al., 2002; Incardona et al., 1998; Lipinski et al., 2008). Remarkably – though not entirely serendipitously – human genetic studies published around that time revealed the first gene associated with HPE: sonic hedgehog (Roessler et al., 1996). Collectively, over five decades, these studies in flies, sheep, mice and humans directly linked Shh signaling to craniofacial birth defects and highlighted the sensitivity of the pathway to both genetic and environmental disruption.

Shh signaling is a logical focus for investigations of etiologically heterogeneous craniofacial birth defects; Shh pathway mutations have been linked to human HPE, and both natural and synthetic inhibitors of Shh signaling have been shown to cause HPE and isolated OFCs in mice. Human malformation-associated mutations have been reported in the *SHH* gene itself, the genes encoding the SHH secretory protein (*DISP1*), the SHH receptor (*PTCH1*) and associated membrane proteins (*CDON*, *BOC* and *GAS1*), and *GLI2*, the dominant pathway transcriptional activator (Hong and Krauss, 2018; Roessler et al., 2018). With respect to environmental influences, the Shh pathway is also inherently sensitive to small molecule modulation. Following the discovery of cyclopamine, numerous small molecules have been identified as pathway inhibitors acting through the same smoothened-targeted mechanism (Pietrobono and Stecca, 2018; Rimkus et al., 2016). Such small molecules include diverse environmental chemicals, such as phytocannabinoids (Khaliullina et al., 2015) and a widely used pesticide synergist, piperonyl butoxide (Everson et al., 2019). Alcohol (ethanol), a known human teratogen, can also be added to this mix and has been suggested to act on multiple factors upstream of and within the Shh signaling cascade to disrupt development of the face and brain (Sulik, 2005). These examples illustrate how a single developmental pathway can be susceptible to a diverse cast of genetic and environmental influences that, individually, may have only subphenotypic impacts but, in combination, produce an additive or synergistic outcome. Teasing apart these interactions is a major research challenge, but one that will be crucial for solving the puzzle of complex birth defects.

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Combining genetic tractability with environmental sensitivity, the mouse is a powerful model to examine specific gene-environment interactions (Hong and Krauss, 2018). *SHH* mutations, the first and most commonly identified in human HPE, provide an instructive example. In human pedigrees, *SHH* mutations are heterozygous and display incomplete penetrance and highly variable expressivity (Solomon et al., 2012). In mice, loss of both *Shh* alleles results in severe HPE, whereas heterozygous mice are indistinguishable from wild-type littermates. However, studies have shown that these 'silent' mutations dramatically exacerbate the teratogenicity of environmental chemicals, including ethanol and piperonyl butoxide (Everson et al., 2019; Kietzman et al., 2014). Similar experiments have shown additive or synergistic interactions between additional gene-environment pairs including *Cdon* and ethanol (Hong and Krauss, 2012), *Gli2* and ethanol (Kietzman et al., 2014), and *Gli2* and the synthetic smoothened antagonist vismodegib (Heyne et al., 2016).

Of course, gene-environment interactions during development are not limited to craniofacial malformations or mediated only through the Shh signaling pathway. Specific gene-environment interactions have been described in mouse-based studies of congenital conditions, including heart disease (Chapman et al., 2019; Moreau et al., 2019), scoliosis (Sparrow et al., 2012), hypospadias (van der Zanden et al., 2012), and complex developmental defects and miscarriage (Cuny et al., 2020; Shi et al., 2017). Importantly, several of these examples go beyond chemicals to demonstrate roles for other environmental influences, including maternal hypoxia (Chapman et al., 2019; Moreau et al., 2019; Sparrow et al., 2012) and nutrient deficiency (Cuny et al., 2020; Shi et al., 2017). Collectively, these findings provide crucial proof of concept for gene-environment interactions in diverse etiologically complex birth defects. However, also apparent in these examples is an inherent limitation of this approach: being time and resource intensive, mouse-based experiments are typically limited to a small 'cherry-picked' list of known factors.

New approaches to solving the gene-environment puzzle

The primary barrier to investigating gene-environment interactions is the sheer number of possible combinatory permutations between the genome and the growing list of chemicals that comprise the exposome, 'the comprehensive characterization of an individual's lifetime exposure history' (Wild, 2011). Although successfully utilized as proof of concept in multifactorial etiologies, mouse studies are not ideally suited for high-throughput discovery of environmental influences and novel interactions. By contrast, zebrafish, although less representative of human development, are increasingly being used to examine gene-environment interactions in high-throughput systems (Balik-Meisner et al., 2018), as discussed in the context of abnormal development in recent reviews (Grinblat and Lipinski, 2019; Krauss and Hong, 2016). Traditional mammalian cell culture approaches offer even greater throughput than zebrafish, but such systems frequently lack the physiological relevance to probe the dynamic cellular and molecular interactions that drive tissue and organ development.

Situated between traditional two-dimensional cell monocultures and animal models are advanced *in vitro* approaches, such as organoids and microphysiological models (MPMs, discussed below), that blend genetic tractability and scalability with varying degrees of physiological complexity. The implementation of *in vitro* systems capable of probing disruptions in developmental processes requires the use of representative cell types, appropriate cellular organization, stability and robustness, methods for assessing function and phenotype, and reproducibility. To test gene-environment interactions, these models must also be genetically tractable. Utility of organoids, three-dimensional (3D) aggregates of self-organized cells, has already been demonstrated in research on the brain, eye, gut, reproductive system, kidney, lung and pancreas (Truskey, 2018). For example, one research group recently demonstrated that brain organoids exposed to a gradient of SHH protein show *in vivo*-like gene expression patterns and that these patterns are disrupted, and organoid growth limited, by Shh signaling inhibition (Cederquist et al., 2019). Organoids have also been utilized to simulate fusion of the human embryonic palate and demonstrate that chemical inducers of cleft palate can inhibit palate organoid fusion *in vitro* (Belair et al., 2018; Wolf et al., 2018). Such models, if appropriately sensitive and scalable, may serve as *in vitro* platforms for the discovery of environmental toxicants with the potential to contribute to OFCs and HPE and other neurodevelopmental abnormalities. Furthermore, utilizing patient-derived cells and leveraging CRISPR/Cas-9 gene-editing technology in developing organoid models expands the utility of this technology in gene-environment interactions research (Truskey, 2018). Organoids should be useful for examining both genetic and environmental disruption of intercellular signaling and tissue organization, at least at the level of the functional unit of an organ. Looking beyond brain and palate, heart and liver organoids have a tendency to resemble embryonic tissue (Takebe et al., 2013; Voges et al., 2017), making them especially promising for mechanistic studies of congenital defects. Organoids have also recently been shown to be amenable to chemical screening approaches (Mills et al., 2019), and efforts are underway to enhance the production and reproducibility of organoids and to develop methods for assessing organoid function (Arora et al., 2017; Kratz et al., 2019).

In contrast to organoids, MPMs, which encompass a broad category of 3D culture models including organ-on-a-chip systems, employ microfabrication and microfluidics to create extracellular structures that aid replication of tissue architecture and physiological forces (Truskey, 2018). One of the goals of MPMs is to simulate a more realistic external environment, for example by providing scaffolding to seed distinct cellular layers (similar to those comprising the cerebral cortex), fluid dynamics to mimic the flow of interstitial fluid within the brain, and microhole structures that simulate the blood-brain barrier (Yi et al., 2015). The use of microfluidics may also produce more realistic exposure scenarios than traditional cell culture models by dynamically controlling the inflow and outflow of treatments or culture components. Furthermore, by combining modular organ-on-a-chip platforms using microfluidics, a degree of xenobiotic metabolism may be incorporated, though scaling is considerably more difficult in complex arrays (Truskey, 2018). Regardless, as these systems typically mimic only a subset of features of an *in vivo* biological system, they are well suited to screening chemicals and, ideally, complex mixtures. In addition, many of the advantages of organoids also apply to MPMs. Genetic tractability, the use of patient-derived cells and 'organoid-on-a-chip' MPM systems that utilize self-organized 3D structures (Skardal et al., 2020) all demonstrate the flexibility and broad potential of modern *in vitro* techniques to improve the faithful recapitulation of biological and physiological processes. In this way, organoids and MPMs may be ideal for the practical detection of toxicants in the environment using an unbiased approach that moves from demonstration of perturbation to chemical identification to mechanistic studies.

Although organoids and MPMs provide an exciting avenue for discovery of gene-environment interactions, their practical limitations must be taken into consideration. Being more complex

than traditional cell culture, advanced *in vitro* models are generally more time and resource intensive, although this gap should narrow as technologies improve. More crucial for the study of birth defects, these advanced cell-based systems do not fully recapitulate dynamic and transient developmental processes or the full physiological complexity of maternal-embryo interfaces, including xenobiotic metabolism and placental transfer. However, advanced *in vitro* systems are not intended to replace all other approaches to birth defects research; instead, organoids and MPMs complement genomics and animal-based models by providing a scalable, human tissue-specific platform for screening gene-environment interactions in complex developmental etiologies.

Perspectives

As organoid and MPM approaches mature, the barriers to high-throughput gene-environment interaction testing will recede. The immense volume of possible interactions becomes less daunting as animal-free models provide insight into the multifactorial mechanisms of physiological disruption in etiologically complex diseases, isolated in a dish from the uncontrollable confounding factors inherent to *in vivo* studies. Once sufficiently scaled, these advanced *in vitro* approaches will allow for more agnostic environmental toxicant screening on customizable genetic backgrounds and biological platforms. This is the modern path to gene-environment interaction discovery. However, for all their benefits, these emerging *in vitro* technologies are not without important drawbacks. The discoveries made in these *in vitro* systems must still be validated in traditional mammalian animal models.

The introduction of advanced molecular and genetic approaches signaled a momentous shift in developmental biology at the turn of the 21st century. The introduction of advanced cell culture techniques may prove equally momentous. Regardless, organoids and MPMs are tools, just as animal models and molecular techniques are tools. Each possesses potential as well as limitations. One approach need not supersede another; rather, we must use all the tools available to us to best serve the wellbeing of those who entrust us with this important research. F. Clarke Fraser opined that birth defects 'are caused by a little bit of this and a little bit of that' (International Neural Tube Defect Conference, 2009). Our approach to solving them, also, must consist of a little bit of this and a little bit of that.

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Competing interests

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