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EPIGENETICS OF TWINS

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Review

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Summary. Epigenetics as a cellular phenomenon represents a variety of cellular processes that regulate chromatin structure and gene expression without changing DNA sequence in a genome. Central epigenetic mechanisms are DNA methylation, posttranslational histone modifications and RNA interference. Epigenetic modifications interact with each other and create a unique epigenome extending throughout the cell, from DNA itself in the nucleus to the cell membrane, and in the context of a multicellular organism throughout a whole organism *per se*. Epigenetics functionally represents a complex molecular bridge that connects genotype and phenotype. Namely, a genome is a rigid structure of encoded inherited traits that determines all possibilities and limitations of a single cell phenotype capabilities, and therefore phenotype of an individual as well. However, in the phenotype, at a given point at a particular site, only certain characteristics encoded in the genome are expressed, and most preferably those that represent the best possible response of a cell and thus of the organism to the requirements of the cellular environment or the environment of the organism *in toto*. Epigenetic mechanisms are dependent on environmental stimuli and strongly affect phenotype properties. In this review, apart from introducing epigenetics, a number of concepts and evidence of interaction between the environment and genotype bridged by epigenetics are mentioned, with highlight on the influence of epigenetics on shaping the phenotypes, especially in monozygotic twins.

Introduction to epigenetics

Epigenetics represents all cellular mechanisms leading to reversible changes in gene expression and chromatin structure that do not involve any change in a sequence of DNA *per se* (1). Indeed, "epigenetic" as a word literally means "around genetic sequence" and therefore has to be differentiated from genetics, well-known genetic laws and genetic way of thinking overall. C.H. Wadington, a British scientist who in 1942 tried to explain the process of a genotype manifesting itself in a phenotype of an individual cell and organism during developmental processes, has coined the term. He hypothesized and described epigenetics as a molecular bridge between genotype and phenotype (2). Since then, many conceptual improvements and breakthroughs have been reached making epigenetics one of the most prominent and promising biological field especially in the frame of biomedical research and cutting edge medical practice (3–5).

DNA sequence, *i.e.* genome, by its function is a "library of inherited properties" and has to remain unchanged throughout a lifespan of an individual organism, meaning single cells as well. Therefore, a genome of an individual has to be "solid" and prone to mechanisms by which it may resist challenges of an environment. Many cellular mechanisms including DNA repair machinery have appeared throughout evolution with the only goal of fighting changes and degradation of inherited features encoded in the DNA sequence. Consequently, a genome is not the one that may provide to a cell, and therefore to an organism as a whole, adaptability to ever-changing requirements of the environment or indeed to demands of a system such a multicellular organism is in fact. On the other hand, epigenetics represents a dynamic mediator between a genotype and corresponding phenotype. Epigenetics is an elegant system enabling to a cell, therefore an organism, to control elements of a genome *i.e.* different treats, to be reversibly projected in a phenotype of a specific cell according to organism demands and environmental challenges in a particular point of time and space (6). In short, stable genome encodes everything that a cell *i.e.* organism may be from fertilization to its death according to inherited traits, while epigenome constantly "filters" all these possibilities and projects in the phenotype only those traits that presents the best possible answer to specific demands of pertaining organism and challenges of organism's environment (7).

Apart of its crucial role in shaping organism's phenotype, the power of epigenetics is best seen on a cellular level. Namely, in a multicellular organism, the epigenetics is indeed organizer of cell phenotype enabling to different embryonic and adult cells to express specific genes/traits that are required for the existence of each cell type. Since epigenome is mitotically inheritable, this phenotype is transferred to daughter cells from which can be transferred further on along the cell lineage, or eventually changed. Noteworthy, these changes can be transferred to the next generation of offspring if occur in germ cells that transfer specific epigenome to a zygote. Paramutation, bookmarking, imprinting, gene expression, X chromosome inactivation, position effect, changeable disorder or phenotypic severity, reprogramming, maternal attributes, carcinogenic processes, teratogenic effects, genomic stability, heterochromatin states and cloning for example are known to involve epigenetic mechanisms (7–9).

Due to very loose definition, many various phenomena have been suggested and are still debated to be part of epigenetics. Still, three of them are accepted as central epigenetic mechanisms; DNA methylation, post-translational histone modifications and RNA interfer-

ence. These cellular phenomena are well described and are in the focus of almost every epigenetic research.

DNA methylation

First described and best understood epigenetic modification is certainly DNA methylation. It represents a quit simple biochemical process of covalent addition of methyl group to the 5th carbon atom of cytosine ring primarily in cytosine-guanine dinucleotide (CpG). Modified by methylation, cytosine converts to 5-methylcytosine (5mC), also called the "fifth base". In mammals, 60–90% of all CpGs are methylated with the exception of CpG rich areas of a genome referred to as CpG islands. Noteworthy, CpG islands frequently coincide with gene promoters. DNA methylation in higher eukaryotes is associated with a dense chromatin environment. Consequently, chromatin with hipermethylated DNA is rigid and usually embodies unexpressed genes (10, 11).

DNA methyltransferase (DNMTs) family enzymes perform methylation of DNA. DNMT1 functions as a maintenance methylase, copying the pre-existing methylation marks onto the new strand during replication of DNA. Therefore, DNMT1 is crucial for DNA methylation inheritance or so called epigenetic cell memory. DNMT3A and DNMT3B are *de novo* methyltransferases *i.e.* enzymes that are able to methylate previously unmethylated CpGs (12–14). Their main function is to guide the change of a cell phenotype in response to cell demands or environmental challenges. Other DNMT family members are DNMT2, involved in RNA methylation pathways, and DNMT3L which lacks catalytic activity but acts as a co-factor for the establishment of (non)CpG methylation (15, 16). In contrast to active processes of DNA methylation, DNA demethylation can be achieved both actively and passively. Passive DNA demethylation usually is a consequence of DNMT1 complete absence of function or at least its malfunction. Active DNA demethylation is performed by ten-eleven translocation (TETs) family enzymes that oxidize 5mC to 5-hydroxymethylcytosine (5hmC). Further on, 5hmC may be either passively depleted through DNA replication or actively reverted to cytosine by thymine DNA glycosylase mediated base excision repair (17).

Apart mechanisms involved, DNA (de)methylation is a crucial force shaping the chromatin and presenting a genome into a phenotype. In fact, it is integrated in every biological segment of individual's life: reproduction, development, management of homeostasis, immunity, healing and regeneration, behavior and social interactions, ageing and in the end death of an organism itself (18–27).

Congruently to mentioned above, according to modern science it is quite unreasonable to expect any disorder or disease not to involve DNA methylation, at least at some extent (28). Maybe the best example of a disease driven by disruptions in DNA methylation is cancer. Indeed, more than enough evidence has been presented for describing cancer as a DNA methylation dependent *i.e.* epigenetic disease (1, 6, 9, 29). In line with these findings, a huge effort is put on developing new, or translating already existing, DNA methylation based diagnostic technology and therapeutic agents in everyday medical practice (28, 30–34).

Histone modifications

Post-translational histone modifications (PTHM), sometimes not quite correctly referred to as *histone code* (35), are a group of primarily covalent additions and eliminations performed at histone tails in nucleosomes (36). All PTHM are executed by enzymes and are highly regulated. In general, PTHM shape the chromatin making it loose and prone to transcription or condensed and transcriptionally silenced (36). Still, comprehensive interpretation of all possible combinations of PTHM is far then simple and straightforward (35). PTHM change the behaviour of a chromatin not just by changing its chemistry and physics. They also recruit various DNA binding proteins as well as remodeling enzymes, which are further reorganizing a chromatin in a specific way influencing *e.g.* gene transcription or DNA repair, replication and recombination (35–38). Noteworthy, PTHM are closely related to DNA methylation, and these two epigenetic modifications tightly collaborate in organizing a genome and shaping the phenotype (39, 40).

One of the simplest, but seemingly most powerful PTHM is enzymatic addition or elimination of acetylic group to histone tail lysines. This PTHM is known as histone (de)acetylation. Histone acetylation neutralize the lysine's positive charge. Thus, it becomes more negative. Since DNA is negatively charged as well, following simple physical law DNA tries to "escape" the nucleosom. This negative-DNA-negative-histone interaction results in a loose chromatin, meaning revealed DNA sequence and more space in a chromatin to place DNA binding proteins such as transcription complex. On the other hand, by eliminating lysines acetylation, histone turns back to positive state and consequently trap DNA more tightly in a nucleosome. Structured like this, chromatin attracts proteins that further compact it leading to highly condensed and transcriptionally silenced heterochromatin (41).

Histone (de)acetylation as an epigenetic mechanism, including biochemistry of enzymes involved and biological repercussions on genome status as well as cell phenotype, is mostly well understood. Regarding its impact on a phenotype, histone (de)acetylation tails DNA methylation and is likewise integrated in every biological segment of individual's life (42).

Similar as with DNA methylation, histone (de)acetylation is under vivid attempt of translating it in clinical practice especially into a therapy of various human disorders and diseases (33, 43–45).

Contrary to histone (de)acetylation, other PTHM *i.e.* histone (de)methylations, (de)phosphorylation, deimination, ADP ribosylation, β-N-acetylglucosamine modification, ubiquitylation and sumoylation along with histone tail clipping and histone proline isomerization are mostly yet to be comprehensively described and their biological impact further acknowledged (36).

RNA interference

RNA interference (RNAi) is the most recently discovered main epigenetic modification (46), for which The Nobel Prize in Physiology or Medicine was awarded in 2006. RNAi is probably the most dynamic epigenetic pathway prone to rapid turnovers (47) with notable impact on gene expression and therefore phenotype itself (48). The real power of RNAi upon shaping a phenotype is presented in the fact that it regulates the expression of around 60% of human genes (49).

Interestingly, it seems RNAi appeared as a primitive but affective cellular immune system mainly against viral genomes. Later on, at some point in eukaryotes evolution, cells started to use the same mechanism not only for defending themselves from exogenous but rather endogenous genetic material. By chance, powerful epigenetic mechanism regulating presentation of traits from a genotype into a phenotype, came into being (50).

MicroRNA (miRNA) and small interfering RNA (siRNA), all in fact non-coding RNAs, are holders of RNA interference machinery and share many similarities. Both are short duplex RNA molecules that exert gene silencing effects at the post-transcriptional level by targeting messenger RNA (mRNA). Both share same enzymatic machinery including proteins like DICER and ARGONAUT as well as RNA induced silencing complex (RISC) (51, 52). Still, crucial difference is that miRNAs have multiple mRNA targets regulating expression of many genes while siRNAs are highly specific focused on targeting precise mRNAs and consequently expression of given genes (53).

As one of three canonical epigenetic modification, RNAi seems to be involved in in each and every cellular phenomena. Consequently, RNAi disruptions may be found as drivers or passengers in various human diseases (54, 55).

Environment, diet and lifestyle

The concept of diet, environment and lifestyle having impact on individual's health and disease is not something new. Still, the scientific fact that all of them have strong and persistent molecular mechanisms to mess up with our genes and changing their expression in our phenotype is quite a novelty (7, 56), and not necessarily a good one (57). Added to mentioned that our decisions today regarding our lifestyle and environment may, first by epigenetic reset followed by epigenetic inheritance through our germ cells, shape a phenotype of our offspring that have yet to come in more than hundred years from now (58), epigenetics suddenly sounds more like an evolutionary curse rather than interesting and peculiar cellular phenomena. Well, although it all may sounds depressive, there is hope enclosed in the definition of epigenetics itself; it is reversible!

While the latest paragraph may sound more poetic than scientific, it actually represent a platform on which new concepts of human disease prevention and health support are tried to be built on. For example, sulforaphane (SFN) which is usually found in cruciferous vegetables such as broccoli, when consumed in a diet for 21 days, suppressed the growth of human prostate cancer cells by 40% in male nude mice due to significant decrease in histone deacetylase activity (59). In the same study, authors showed that in human subjects a single dose of 68 g BroccoSprouts inhibited histone deacetylase (HDAC) activity significantly in peripheral blood mononuclear cells 3 and 6 hours following consumption. These findings provide evidence that one mechanism through which SFN acts as a cancer chemopreventive agent *in vivo* is through the inhibition of HDAC activity (59). Another research, designed as a year-long lifestyle modification program, was conducted to mediate cardiovascular risk through traditional risk factors and to investigate how molecular changes may contribute to long-term risk reduction. According to obtained results, authors concluded that successful and sustained modulation of gene expression through healthy lifestyle had beneficial effects on vascular health and reduction of traditional risk factor profiles (60). In a pilot trial, stress reduction by transcendental meditation and extensive health education reduced patients' blood pressure by, according to authors, change in telomerase gene expression (61). Without discussing underlying molecular mechanisms, this study indisputably showed that in fact even by a simple meditation, one is able to change gene expression profile with consequent phenotype modification.

In short, one of the most exciting features of using epigenetics reversibility as well as dynamicity, through modulating lifestyle, environmental quality and diet especially, is a potential to offer cheap and responsive alternative ways of preventing or curing diseases to which people are even hereditarily prone, including inflammation, diabetes, obesity, cancer, and neurodegenerative diseases for example (56).

Dizygotic twins

Considering epigenetics, dizygotic twins are perceived as (non)related individuals. They are much more studied in terms of traits in genotype and phenotype they share rather than those that differ them. Still, dizygotic twin share at least intrauterine environment as well as time and place of their existence if not organized differently (62, 63). This indeed cannot be ignored in term of comparing their health and disease statuses *i.e.* genotype relation to phenotype through epigenetics under environmental influence, as described above *(Figure 1)*.

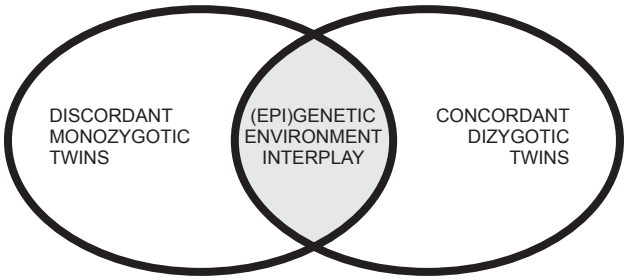

Figure 1. Benefits from twin studies in understanding epigenetics. Studying discordant monozygotic twins, impact of environment on a phenotype by induced epigenome rearrangements of individuals with identical genome, may be elucidate. Taking in account differences in genomes, contrary may be identified in concordant dizygotic twins sharing same traits. Combining these approaches, much can be understood about environment conditioning genome presentation in phenotype by modulating epigenome.

Monozygotic twins

Monozygotic twins provide a simple natural system for studying epigenetic plasticity as well as epigenetic role and power in shaping a phenotype including phenotypes of various disorders and diseases.

Although monozygotic twins are believed, not quite correctly, to share identical genome, they do not have to necessarily shape the same phenotype due to differences in exposed environment *(Figure 2)*. This fact usually is not perceived for *in utero* period of twin's life. Indeed, it should. Namely, some of *in utero* events or condition, such as unequal division of blastomeres or uneven vascularization of the placenta, can be considered as non-shared early environmental exposures. It is well known that *in utero* growth restriction is in fact more pronounced in monozygotic twins. Differences in placental sharing and vascularization lead to occasional unequal blood and nutrient sharing, and, in about 15% of monozygotic twins diamniotic pregnancies result in twin-to-twin transfusion syndrome (64). Indeed, a major difference in the intrauterine environment between monozygotic twins seems to be whether they share, or not, a common placenta. Using DNA methylation measured at >400,000 points in the genome, it was demonstrated that the co-twins of monozygotic pairs (average age of 14) that shared a common placenta have more similar DNA methylation levels in blood throughout the genome relative to those with separate placentas. Functional annotation of the genomic regions that show significantly different correlation between monochorionic and dichorionic pairs found an over-representation of genes involved in the regulation of transcription, neuronal development, and cellular differentiation. These results support the idea that prenatal environmental exposures may have a lasting effect on an individual's epigenetic landscape, and the potential for these changes to have functional consequences (65).

Monozygotic twins have a higher incidence of congenital heart disorders, and this noteworthy usually occurs in one twin only (64). The higher risk nature of multiple pregnancies, their proclivity towards complications, and the twin-twin competition for maternal resources increases the probability of a skewed environment affecting the twins in utero respectively (64), changing their phenotype regardless of sharing identical DNA sequence.

After birth, any non-shared environmental exposure, such as diet, smoking, toxin exposure and infection, as mentioned, may contribute towards twin discordance (64, 66, 67). One study examined the global and locus-specific differences in DNA methylation and histone acetylation of a large cohort of monozygotic twins. It found that, although twins were epigenetically indistinguishable during the early years of life, older monozygotic twins presented remarkable differences in their overall content and genomic distribution of methylated DNA and histone acetylation. These epigenetic differences affected their gene-expression portrait and phenotype overall (67).

Other study investigated if serum microRNAs (miRs) could explain discordance in non-alcoholic fatty liver disease (NAFLD). A cross-sectional analysis of a prospective cohort study was performed in 40 twin pairs. All participants underwent a standardised research visit, liver MRI using proton-density fat fraction to quantify fat content, and miR profiling of their serum. Results showed 6 concordant twin pairs for NAFLD, 28 concordant for non-NAFLD while 6 were discordant for NAFLD. Within the six discordant twins, a panel of 10 miRs differentiated the twin with NAFLD from the one without. Two of these miRs, miR-331-3p and miR-30c, were also among the 21 miRs that were different between NAFLD and non-NAFLD groups. Both miRs were highly heritable and highly correlated with each other suggesting involvement in a common mechanistic pathway. An interactome analysis of these two miRs showed seven common target genes. In conclusion, this research demonstrated that discordancy in liver fat content between the twins can be explained by miRs, and that this discordany seems to be heritable (68).

To make this (epi)genome environment interplay in shaping a phenotype more complicated, there are reasonable evidences that already established phenotypic differences arising in twins could potentially cause shared exposures to have different effects, leading to further dissimilarity between the twins (67).

Organism's phenotype present at a specific time relay on epigenetic responses to environmental and lifestyle challenges encountered by the organism itself. Still, this epigenetic response modelling the phenotype is possible only in the frame of inherited traits in the genome. Left side of the picture presents a (non)related individuals scenario, where organisms differ from each other by the inherited genome and therefore diapasons of possible epigenetic answers i.e. phenotypes, in the start. Reasonably, this scenario will result in different phenotypes between individuals even if challenged by the similar environment or lifestyle. Epigenetic force shaping a phenotype is sometimes referred to as epigenetic drift (69). Right side of the picture presents monozygotic

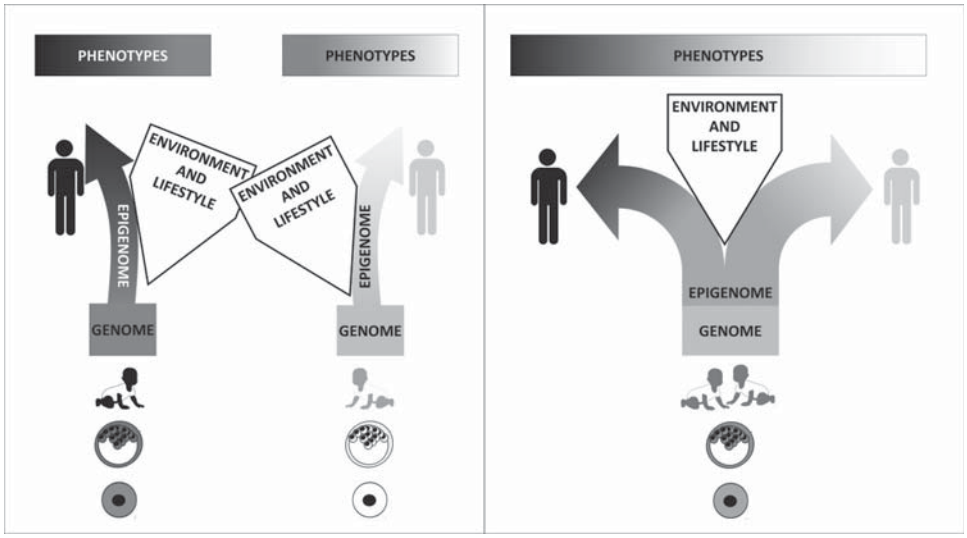

Figure 2. Epigenetic drift and epigenetic wedge.

twins scenario where all individuals inherited the same genome and therefore diapasons of possible phenotypes. Although it would be expected them to shape the same phenotype, they rather accumulate differences throughout their life, including in utero period. Developed differences in their phenotypes relay on either different epigenetic response to environment and lifestyle or even slightly but still present differences in environment and lifestyle, to which they are exposed, per se. Indeed, stronger differences in environment and lifestyle encountered by the twins, stronger differences in their epigenome and therefore phenotypes will appear in time. We suggest referring to this epigenetic force as to *epigenetic wedge* since differences in shaping phenotypes, although sharing the same genotype, are way more dramatic.

Conclusion

This review is just a preview indeed, of what can be reasonably expected from epigenetics to represents in fact for biology and biomedicine of monozygotic and dizygotic twins. Therefore, it should not surprise why authors from the field highlight how an appreciation of epigenetics is missing from the understanding of twin phenotype organization phenomena i.e. biological process of shaping different phenotypes originated from the same genotype (67).

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EPIGENETIKA BLIZANACA

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Pregled

Ključne riječi: epigenetika, genotip, fenotip, okoliš, blizanci

Sažetak. Epigenetika kao stanični fenomen, okuplja niz različitih staničnih procesa koji reguliraju strukturu kromatina te gensku ekspresiju bez zadiranja u sekvencu genoma. Središnji epigenetički procesi svakako su metilacija DNA, posttranslacijeke histonske modifikacije i RNA interferencija. Navedene epigenetičke modifikacije međusobno interagiraju i stvaraju jedinstveni epigenom koji se prostire kroz čitavu stanicu, od same DNA u jezgri do stanične membrane, a u kontekstu multicelularnog organizma kroz cijeli organizam *per se*. Epigenetika funkcionalno predstavlja kompleksan molekularni most koji spaja genotip i fenotip. Naime, genom je rigidna struktura ukodiranih naslijednih svojstava koji određuju sve mogućnosti i ograničenja fenotipa stanice i jedinke. Međutim, u fenotipu se u danom trenutku na određenom mjestu eksprimiraju samo određena svojstva kodirana u genomu, i to ponajprije ona koja predstavljaju najbolji mogući odgovor stanice, a time i organizma u cijelosti, na zahtjeve staničnog okoliša odnosno okoliša jedinke *in toto*. Epigenetički mehanizmi ovisni su o utjecaju okoliša i snažno utječu na isplovljavanje svojstava u fenotipu. U ovom preglednom radu, pored uvoda u osnove epigenetike, navodi se niz koncepata i dokaza o interakciji okoliša i genotipa preko epigenetike s naglaskom na spoznaje o utjecaju epigenetike na modeliranje fenotipa posebice monozigotnih blizanaca.