

AOP ID and Title:

AOP 443: DNA damage and mutations leading to Metastatic Breast Cancer
Short Title: DNA damage and metastatic breast cancer

Graphical Representation

Level of Organization	AOP Diagram
Macro-molecular	Increased DNA damage and mutation inadequate DNA repair increased mutations increased miRNA expression decreased SIRT1 expression increased activation of NF κB Increased chromosomal aberrations
Cell/Tissue	Induction, Epithelial Mesenchymal Transition Antagonism, Estrogen receptor
Organ/Organ System	Metastatic Breast Cancer
Individual	Females Metastatic Breast Cancer

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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.103	Included in OECD Work Plan

Abstract

This adverse outcome pathway details the effect of alcohol as a stressor in metastatic breast cancer. Aim of this AOP is intended to detail the linkage between alcohol and miRNA- SIRT-1 axis induced metastatic breast cancer which represents a knowledge gap as there are not many references available. Consecutive KEs identified are as follows.

Acetaldehyde, which is a metabolite of alcohol is considered a major mutagen which has been determined to induce genotoxic effects on DNA resulting in increased DNA damage. Inadequate DNA crosslink repair mechanisms leads to accumulation of damaged DNA resulting in impaired DNA synthesis leading to mutations and increased miRNA expression ; leads to disruption of SIRT-1 signalling . This step is followed by increased acetylation and activity of NFκB ; loss of estrogen receptor functions ; molecular alterations of epithelial cells ; gain of mesenchymal cell features ; eventuating in increased invasion and migration of breast cancer cells resulting in Metastatic breast cancer .

Background

Alcoholic beverages are classified by the International Agency for Research on Cancer(IARC) as Group 1 carcinogens. Studies have reported alcohol consumption to be a risk factor for breast cancer in women(Room R et al, 2005). A woman drinking an average of two units of alcohol per day has an 8% higher risk of developing breast cancer than a woman who drinks an average of one unit of alcohol per day[2]. Alcohol is metabolized by alcohol dehydrogenase to acetaldehyde which is a mutagen. Various theories have been proposed which explain the mutagenicity of alcohol. Among them, the most relevant one for carcinoma of the breast has been proposed by Purohita et al, suggesting an alcohol-induced inactivation of the tumor suppressor gene BRCA1 and increased estrogen Responsiveness in breast tissues(Purohit V et al, 2005). Boffetta and Hashibe list plausible mechanisms of breast cancer as a result of the genotoxic effect of acetaldehyde-induced increased estrogen concentration(Boffetta P et al 2006). It has also been found that alcohol stimulates the epithelial-mesenchymal transition (EMT), because of which there is distant metastasis (Forsyth C. B. et al 2010). However, this mechanism needs to be elucidated in detail.

MicroRNAs (miRNAs) are non-coding, single-stranded RNA molecules that regulate target gene expression via post-transcriptional modifications [Mohr A. M& Mott J. L 2015 and Lai E. C. 2002]. Several studies indicated the promising role of miRNA in the diagnosis and outcome prediction in several cancers (Mirzaei H et al 2018 and Liu, S. Y et al 2017). miRNA-21 is upregulated and promotes metastasis in several cancers (Kunita, A et al 2018 and Liu Z et al 2015). A Chinese study by Kunita et al proved that plasma levels of miRNA were up-regulated in large B-cell lymphoma patients (Kunita, A et al 2018). A study by Wang et al also proved that plasma levels of miR were upregulated in large B-cell lymphoma patients in China (Chen et al 2014). Although miR-21 was indicated to play a crucial role in the metastasis of lung cancer, ovarian cancer, and head and neck cancer through several signaling pathways, the molecular mechanism of how miR-21 regulates the EMT process in breast cancer is not clear (Liu S. Y et al, Lopez-Santillan et al 2018, Panagal M. et al 2018, Zhou, B.et al 2018, Brabletz T et al and Ye, X. et al 2017).There are a number of miRNAs which regulate SIRT 1 expression. The epithelial-mesenchymal transition (EMT) is a process that which epithelial cells lose their cell polarity and cell adhesion ability, which will lead to cancer metastasis (Vaziri H et al 2001 and Luo, J et al 2001). Epithelial cells exhibit the property of regular cell-cell contacts, adhesion to the surrounding cellular fabric, preventing the detachment of individual cells. Whereas mesenchymal cells do not form intercellular contacts.

Sirtuins are nicotinamide adenine dinucleotide (NAD⁺)–dependent deacetylases that function as intracellular regulators of transcriptional activity (Blander G & Guarente L 2004 and Roth M & Chen W 2014). It plays important roles in cell survival, signal transduction, and cell apoptosis by deacetylating key cell signaling molecules and apoptotic related proteins, such as NF-κB, p53, Ku70, and HIFs (Zhao, W et al 2008 and Chen W & Bhatia R 2013). Various studies have inconclusive reports on the role of SIRT1 in cancer, because of its opposite effects as both a tumor activator or suppressor in various human cancers, including breast cancer. Deng et al found that the expression of SIRT1 was lower in prostate cancer, bladder cancer, ovarian cancer, and glioblastoma when compared with normal tissues (Han, L et al 2013).On the contrary, it was found that, in leukemia and lung cancer, SIRT1 was significantly higher(Riggio M et al 2012 and Lee M S et al 2015).

This can be explained as follows: SIRT1-mediated deacetylation suppresses the functions of several tumor suppressors including p53, p73, and HIC1, it has been suggested that SIRT1 has a promoting function in tumor development and progression [Pinton G et al 2016, Pillai VB et al 2014, Wan G et al 2017 and Hwang B et al 2014]. In contrast, SIRT1 may have a suppressive activity in tumor cell growth by suppressing NF-κB, a transcription factor playing a central role in the regulation of the innate and adaptive immune responses and carcinogenesis, the dysregulation of which leads to the onset of tumorigenesis and tumor malignancy(Yuan J et al 2009, Wang R H et al 2008, Chen L F et al 2004 and Greten F R & Karin M 2004). Here, we aim to further explore the role of the SIRT1-NF κB signaling pathway in tumorigenesis of the breast as well as its associated mechanisms.

The nuclear factor-κB (NF- κB)/REL family of transcription factors is comprised of a RELA/p65,c-REL, RELB, p105/NF- κB1 and p100/NF- κB2 (Van Laere S J et al 2007). The p105 and p100 proteins can be processed by proteolytic cleavage into p50 and p52, respectively. Activation of the NF-κB signaling pathway leads to the induction of target genes that can inhibit apoptosis, interaction with cell cycle regulation, cell

invasion, contribute to tumorigenesis and metastatic invasion (Shostak K & Chariot 2011). Activation NF-κB in breast cancer is loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Factor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen-Activated Protein Kinase (MAPK) pathway (Ali S & Coombes R C 2002). Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) also ultimately activates NF-κB and most likely contributes to the enhanced activity of this transcription factor in ER-negative breast cancer cells (Kalkhoven E et al 1996).

Loss of ER function has been associated with constitutive NFκB activity and hyperactive MAPK, because of constitutive secretion of cytokine and growth factors, which ultimately culminates in aggressive, metastatic, hormone-resistant cancers (Merkhofer E C et al 2010). Activation of the progesterone receptor can lead to inhibition of NF-κB driven gene expression (Sethi G et al 2008) reducing its DNA binding and transcriptional activity. HER-2 activates NF-κB through the canonical pathway which surprisingly, involves IKKα (Ito, T et al 2010). Activation of NF-κB promotes the survival of tumor cells. Several gene products that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation (Lee J et al 2010). Estrogen plays an important role in breast cancer initiation and progression. Breast cancer over time acquires different mutations and the proportion of estrogen receptor-negative cells in tumors increases. This transformation confers aggressive biological characteristics to breast cancer such as rapid growth, poor differentiation, and poor response to hormone therapy. NF-κB pathway plays important role in this pathway (Lee J et al 2010).

Expression of SIRT1 is controlled at multiple levels by transcriptional, post-transcriptional, and post-translational mechanisms under physiological and pathological conditions. Emerging evidence indicates that miRs are important regulators of SIRT1 expression (Lovis P et al 2008, Ortega F J et al 2010, Zovolis A et al 2011, Yamakuchi M et al 2008 and Mullany L E et al 2017). Studies have shown that miR-34a directly binds to the 3' untranslated region (UTR) of SIRT1 mRNA and reduces its expression (Ortega F J et al 2010).

Study findings support the hypothesis that alcohol consumption is able to influence miRNA expression. Considerable evidence from rodent and human studies demonstrates that disruption of the hepatic SIRT1 signaling by ethanol plays a central role in the development of AFLD (Yin H et al 2014, Li M et al 2014).Ethanol down-regulates SIRT1 in hepatic cells and in the animal livers. The ethanol-mediated disruption of SIRT1 signaling leads to excess fat accumulation and inflammatory responses in the liver of animals and humans. Treatment with resveratrol, a known SIRT1 agonist, can alleviate liver steatosis . Accumulating evidence demonstrates that ethanol-mediated SIRT1 inhibition leads to the development of AFLD largely through disruption of a signaling network mediated by various transcriptional regulators and co-regulators, including nuclear transcription factor-κB (NF-κB)(Yin H et al 2014, Li M et al 2014).

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	1669	Increased, DNA damage and mutation	Increased, DNA damage and mutation
2	KE	155	Inadequate DNA repair	Inadequate DNA repair
3	KE	185	Increase, Mutations	Increase, Mutations
	KE	1554	Increase Chromosomal Aberrations	Increase chromosomal aberrations
4	KE	1980	Increased micro RNA expression	Increase,miRNA levels
5	KE	1981	Decreased SIRT1 expression	Decrease,SIRT1(sirtuin 1) levels
6	KE	1172	Increase activation, Nuclear factor kappa B (NF-κB)	Increase activation, Nuclear factor kappa B (NF-κB)
7	KE	112	Antagonism, Estrogen receptor	Antagonism, Estrogen receptor
8	KE	1457	Induction, Epithelial Mesenchymal Transition	EMT
9	AO	1982	metastatic breast cancer	Metastasis, Breast Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, DNA damage and mutation	adjacent	Inadequate DNA repair	High	High
Inadequate DNA repair	adjacent	Increase, Mutations	High	High
Increase, Mutations	adjacent	Increased micro RNA expression	Moderate	Moderate
Increased micro RNA expression	adjacent	Decreased SIRT1 expression	Moderate	Moderate
Decreased SIRT1 expression	adjacent	Increase activation, Nuclear factor kappa B (NF-κB)	Moderate	Moderate
Increase activation, Nuclear factor kappa B (NF-κB)	adjacent	Antagonism, Estrogen receptor	Moderate	Moderate
Antagonism, Estrogen receptor	adjacent	Induction, Epithelial Mesenchymal Transition	High	High
Induction, Epithelial Mesenchymal Transition	adjacent	metastatic breast cancer	High	High
Increased, DNA damage and mutation	non-adjacent	Increase Chromosomal Aberrations	High	High
Increase Chromosomal Aberrations	non-adjacent	Increased micro RNA expression	High	High

Stressors

Name	Evidence
Ethyl alcohol	High

Overall Assessment of the AOP

Increased DNA damage and mutations [Evidence: high]: DNA damage refers to any modification in the physical and/or chemical structure of DNA resulting in an altered DNA molecule that is different from the original DNA molecule with regard to its physical, chemical, and/or structural properties". External factors to the cell such as environmental or potentially aggressive factors produced by the normal cell metabolism can damage the DNA. The effects caused by the action of endogenous factors may be more serious and/or more extensive than the effect of most of the exogenous DNA damaging factors. Evidence suggested that prolonged alcohol intake is positively associated with an increased risk of cancer. It can cause changes in the sequence of genomic DNA, which may act as a tumor promoter as well. Alcohol consumption can result in the generation of DNA-damaging molecules such as reactive oxygen species (ROS), lipid peroxidation products, and acetaldehyde. Strand breaks and oxidative base damage in DNA can be produced by hydroxyl radicals which are both mutagenic and cytotoxic. Alcohol is a known inducer of microsomal oxidizing system, which includes a specific ethanol-inducible form of cytochrome **P450**, referred to as CYP2EL (Lieber C 1992). This effect on the enzyme system has been associated with liver pathology induced by alcohol (Morimoto M et al 1993, French S et al 1993, Nanji A et al 1994, Albano E et al 1996). Again the damaging effects of high levels of CYP2E1 may be mediated by the generation of ROS (Cederbaum 1989, Reinke L et al 1990, Ishii H et al 1989). ROS that is highly reactive, include the oxygen radicals superoxide anion and hydroxyl radicals and can react with lipids, proteins, and DNA and thereby damage them (Knecht K et al 1990). It has been confirmed in vivo experiments that hydroxyethyl radical formation takes place after ethanol exposure (Albano E et al 1996, Moore D et al 1995, Clot, P et al 1996, Thurman R 1973). Chronic exposure to ethanol also results in increased production of H₂O₂, (Kukielka E et al 1992, Kukielka, E., & Cederbaum, A. I. 1994) which can react with metal ions (such as iron in the Fenton reaction); thus resulting in the production of the highly reactive hydroxyl radicals. DNA is very sensitive to the attack by the hydroxyl radical. A sensitive assay for hydroxyl radical formation from CYP2E1 uses DNA damage (strand breakage) as an endpoint (Breen AP, Murphy JA 1995). Apart from this, more than twenty different types of DNA base damage with diverse biological properties are produced by hydroxyl radical (Moriya M 1993).8-hydroxy-2'-deoxyguanosine, is one such DNA lesion brought about by oxidative stress. **This is** mutagenic, due to the tendency of DNA polymerases to misincorporate deoxyadenosine residues opposite this oxidized base (Song B 1996).

Inadequate DNA cross-link repair mechanisms [Evidence:high]:

As a result of DNA damage, DNA repair activities change. A variety of genotoxic agents, such as N-nitrosodimethylamine, aflatoxin B1, and 2-acetylaminofluorene induce the protein, O6-Alkylguanine-DNA alkyltransferase (ATase), are responsible for the repair of DNA alkylation damage in rats (O'Connor, 1989; Chinnasamy et al.,1996). Grombacher and Kaina (1996) reported an increased human ATase mRNA expression by alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine and methyl methanesulphonate and by ionizing radiation via the induction of the ATase promoter. ATase mRNA expression was increased in response to treatment with 2-acetylaminofluorene in rat liver (Potter et al., 1991; Chinnasamy et al., 1996). In another study, it was demonstrated that ATase gene induction is p53 gene-dependent: ATase activity was induced in mouse tissues following γ-irradiation in p53 wild-type mice, but not in p53 null animals (Rafferty et al., 1996). Alkylating agents and X-rays also induce DNA glycosylase, alkylpurine-DNA-N-glycosylase (APNG) (Lefebvre et al., 1993; Mitra and Kaina, 1993).

Increased mutations [Evidence: moderate]: Inadequate repair causes damaged DNA to be retained and used as a template during DNA replication. Incorrect nucleotides may be inserted during the replication of damaged DNA, and these nucleotides become 'fixed' in the cell after replication. The mutation propagates to more cells as a result of further replication. Non-homologous end joining (NHEJ) is one of the repair methods employed in human somatic cells to repair DNA double-strand breaks (DSBs). (Pettrini et al., 1997; Mao et al., 2008). However, this mechanism is prone to errors and may result in mutations during the DNA repair process. (Little, 2000). As it does not use a homologous template to repair the DSB, NHEJ is considered error-prone. Many proteins work together in the NHEJ pathway to bridge the DSB gap by overlapping single-strand termini that are typically less than 10 nucleotides long. (Anderson, 1993; Getts & Stamato, 1994; Rathmell & Chu, 1994). Errors are introduced during this process, which can result in mutations like insertions, deletions, inversions, or translocations.

Increased micro RNA expression [Evidence: moderate]: DNA damage-responsive transcription factors, such as NF-κB, E2F, and Myc, are also involved in miRNA transcription regulation. The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators. For example, p53 prevents the TATA-binding protein from binding to the TAATA site in the promoter of the miR-17-92 cluster gene, suppressing transcription. Under hypoxic conditions, the miR-17-92 cluster is suppressed by a p53-dependent mechanism, making cells more susceptible to hypoxia-induced death (Yan et al.,2009).

Decreased SIRT1 expression [Evidence: moderate]: There are several signaling pathways that establish the role of increased miRNA expression in downregulating the SIRT 1 gene few of which are listed as follows; Butyrate has been demonstrated to cause apoptosis and reduce carcinogenesis in a variety of cancers (Tailor et al.,2014; Rahmani et al.,2002). Although butyrate has been shown to suppress SIRT1 gene expression in various cancers, this has yet to be proven in hepatocellular carcinoma (HCC) (Iglesias et al., 2007). In HCC, miR-22 was found to be downregulated, and its low levels aided carcinogenesis (Zhang et al.,2010). The Huh7 cells' in vitro proliferation was decreased by miR-22 expression, which activated apoptosis. In Huh7 cells, on the other hand, SIRT1 expression was high, which enhanced the expression of antioxidants such as superoxide dismutase (SOD), allowing cell growth to continue (Chen et al.,2012). Butyrate upregulated miR-22 in Huh7 cells, which binds directly to the 3'UTR region of SIRT1 and suppresses its expression.Notch3–SIRT1–LSD1–SOX2 Signaling Pathway in metastasis (Wang et al.,2016; Wu et al .,2017).MiR-486 inhibits HCC invasion and tumorigenicity by directly targeting and suppressing SIRT1 expression. This reduced the tumorigenic and chemo-resistant features of LCSCs and inhibited HCC invasion and tumorigenicity (Yan et al.,2019).

Increased activity of NF κB [Evidence: moderate]: SIRT1 deacetylates NFκB. In the context of NFκB, all of the evidence so far points to its signaling being inhibited after SIRT1 deacetylation (Morris, 2012). According to Yeung et al, SIRT1 can directly interact with and deacetylate the RelA/p65 component of the NF-B complex (Yeung et al.,2004). NF-B can be activated by cytokines (TNF-, IL-1), growth factors (EGF), bacterial and viral products (lipopolysaccharide (LPS), dsRNA), UV and ionizing radiation, reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells. Almost all stimuli eventually activate a large cytoplasmic protein complex called the inhibitor of B (IB) kinase (IKK) complex via a so-called "canonical pathway." The exact composition of this complex is unknown, however, it has three fundamental components: IKK1/IKK, IKK2/IKK, and NEMO/IKK. IB is phosphorylated by the activated IKK complex, which marks it for destruction by the -transducin repeat-containing protein (-TrCP)-dependent E3 ubiquitin ligase-mediated proteasomal degradation pathway (Liu et al., 2012;Li et al., 2002). As a result, unbound NF-B dimers can go from the cytoplasm to the nucleus, bind to DNA, and control gene transcription.

Antagonism of estrogen receptor [Evidence: moderate]: Activation NF-κB in breast cancer leads to loss of Estrogen Receptor (ER) expression and Human Epidermal Growth Factor Receptor 2 (HER-2) overexpressed via epidermal growth factor receptor (EGFR) and Mitogen-Activated Protein Kinase (MAPK) pathway (Laere et al.,2007). Indeed, the binding of epidermal growth factor (EGF) to its receptor (EGFR) activates NF-B, which most likely contributes to this transcription factor's increased activity in ER-negative breast cancer cells (Shostak et al.,2011). Because of the constitutive production of cytokines and growth factors, loss of ER function has been linked to constitutive NF-κB activity and hyperactive MAPK, resulting in aggressive, metastatic, hormone-resistant malignancies (Ali et al., 2002). Activation of the progesterone receptor can reduce DNA binding and transcriptional activity by inhibiting NF-B-driven gene expression (Kalkhoven et al., 1996). HER-2 stimulates NF-B via the conventional route, which includes IKK (Merkhofer et al., 2010).

Epithelial-mesenchymal transition cell [Evidence: high]: Estrogen/ERα signaling maintains an epithelial phenotype and suppresses EMT.ERα signaling promotes proliferation and epithelial differentiation and opposes EMT. Various studies support this finding (Eeckhoutte et al.,2007, Kouroos-Mehr et al.,2008, Nakshatri et al., 2009, Taylor et al.,2010). ER-α negative was related to activation of genes implicated in Wnt, Sonic Hedgehog, and TGF-β signaling, indicating epithelial-mesenchymal transition (EMT)(Wik et al.,2013).

Metastatic breast cancer [Evidence: high]: The "epithelial-mesenchymal transition" (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas in recent years. Some of the cells undergoing EMT have the characteristics of cancer stem cells (CSCs), which are linked to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015). Cancer metastasis and cancer therapeutic resistance are linked to the EMT phenomenon (Smith & Bhowmick, 2016; Tanabe, 2013). EMT causes the cell to escape from the basement membrane and metastasize by increasing the production of enzymes that break down extracellular matrix components and decreasing adherence to the basement membrane (Smith & Bhowmick, 2016).

Overall Assessment:

overall assessment of the AOP was based on the biological domain of the applicability, the essentiality of all KEs, Biological plausibility of each KER, Empirical support for each KER, and Quantitative weight of evidence considerations optional.

	MIE 1669	KE 155	KE 185	KE190	KE191	KE 1172
Sex/Life stage /Taxa	Female/Reproductive/Human,human cell line,mice,rat	Female/Reproductive/Rat/rat cell lines/mouse	Female/Reproductive/Mice, yeast,hman cell line	Female/Reproductive/canine,mouse,human cell line	Female/Reproductive/human,human cell ine	Female/Reproductive/human,hum cell ine,mice
Essentiality of KEs	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence	Direct Evidence
Empirical Support of KER	High for MIE1669 to KE155	High for KE 155 to KE 185	Moderate for KE 185 to KE190	Moderate for KE190 to KE191	Moderate for KE191 to KE 1172	Moderate for KE 1172 to KE 112
Biological plausibility of KER	High for MIE1669 to KE155	High for KE 155 to KE 185	Moderate for KE 185 to KE190	Moderate for KE190 to KE191	Moderate for KE191 to KE 1172	Moderate for KE 1172 to KE 112
Quantitative assessment	PCR-RFLP OHdG – ELISA & RT- PCR, Western Blot MAPK assay, Immunoprecipitation, Western immunoblotting s.	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase.	Acetaldehyde assay, Extract preparation and Western blotting, N ² - Ethyl dGuo quantitation	Western blotting,clonal survival assay,FACs	qRT-PCR,Western blotting, Luciferase reporter assay Micro-array	qRT-PCR,immunohistochemistry
References	Chen CH et al 2011,	Panida Navasumrit et al, 2001),Kotova N et al, 2013, Garaycochea JI et al, 2012	Abraham J et al 2011, Garaycochea JI et al, 2018, Voordeckers K et al, 2020	van Jaarsveld MT et al 2014, Abdelfattah, N et al, 2018,Liu Z et al, 2017,Zhang X et al,2011 Wan G et al, 2013,Bulkowska M et al, 2017	Shen ZL et al 2016,Guo S et al 2020,Bae HJ et al 2014,Zhou J et al 2017,Fu H et al 2018,,Lian B et al 2018,Guan Y et al 2017,Yang X et al 2014,Jiang G et al 2016,Luo J et al 2017,Tian Z et al 2016,Yan X et al 2019,Zhang S et al 2016	McGlynn LM et al 2014,Paul Pfluger et al 2008,Yeung F et al 2004

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI
rat	Rattus norvegicus	Moderate	NCBI
canine heartworm nematode	Dirofilaria immitis	Moderate	NCBI
yeast	Saccharomyces cerevisiae	Moderate	NCBI

Sex Applicability

Sex	Evidence
Female	High

Sex:The AOP is applicable to women.However study suggests that the relative risk of breast cancer in men is comparable to that in women for alcohol intakes below 60 g per day. It continues to increase at high consumption levels not usually studied in women (Guénel P et al 2004).

Life stage:There are no research articles which highlight the role alcohol in a particular life stage.In addition, age-related differences in response to alcohol exposure are neither uniform nor linear. The data available is insufficient which direct the construction of a catalog of "appropriate" tests or to define all the factors which influence nonlinear effects (Squeglia LM et al 2014).

Taxonomic:The evidences for the key events of this AOP are available in various species ike rat,mice and humans.

Essentiality of the Key Events

Direct evidence is available for all the suggested key events. However the strength of weight of evidence varies from moderate to high. however, some inconsistencies are also available. majority of the experimental evidence is available in rats, mice, canine and human cell lines. only a few studies are available on human subjects.

- Human normal hepatocytes (HL-7702) were subjected to escalating doses of N,N-dimethylformamide for 24 hours (C. Wang et al., 2016). At all concentrations, a concentration-dependent increase in ROS was detected; the rise was statistically significant when compared to control (6.4, 16, 40, 100 mM). Until the highest two concentrations (40 and 100 mM), no significant rise in 8-oxodG was seen, indicating inadequate repair at these dosages. Excision repair genes (XRCC2 and XRCC3) were considerably up-regulated at 6.4 and 16 mM, well below the doses that significantly produced 8-oxodG, indicating that

adequate DNA repair was possible at these low concentrations. These findings show that repair is competent at low concentrations (removing 8-oxodG quickly), but that repair is swamped (i.e., insufficient) at larger doses, where 8-oxodG greatly increases. AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008). Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m² UVA irradiation. OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h.Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions. Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m² UVA irradiation.G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.

- There is evidence from knock-out/knock-down studies indicating there is a strong link between DNA repair adequacy and the frequency of mutations. Defects in proteins involved in DNA repair resulted in altered mutation frequencies in all of the instances studied when compared to wild-type cases. In cell lines deficient in LIG4 (Smith et al., 2003) and Ku80 (Feldmann et al., 2000), there were significant decline in the frequency and accuracy of DNA repair; rescue experiments performed with these two cell lines further confirmed that inadequate DNA repair was the cause of the observed decreases in repair frequency and accuracy (Feldmann et al., 2000; Smith et al., 2003). There was more spontaneous DNA damage in Nibrin-deficient mouse cells than in wild-type controls, implying insufficient DNA repair. In vivo mutation frequencies were also observed to be higher in Nibrin-deficient mice than in wild-type mice using the corresponding Nibrin-deficient and wild-type mice (Wessendorf et al., 2014). Furthermore, depending on the XPC status of cancer patients, mutation densities in certain genomic areas were influenced differentially. In XPC-wild-type patients, mutation frequencies were higher at DHS promoters and 100 bp upstream of TSS than in cancer patients missing functional XPC (Perera et al., 2016). Finally,it was found that radiation exposure caused four times more mutations in WKT1 cells with lower repair capacity than in TK6 cells with normal repair capacity in a research (Amundson and Chen, 1996).
- There are findings that strongly link the different elements of DNA damage and repair events to the expression of miRNA. Zhang and coworkers examined genome-wide mature miRNA expression in Atm+/+ and Atm-/- littermate mouse embryonic fibroblasts to see how miRNAs are regulated in the DNA damage response (MEFs)(Zhang et al.,2011). MEFs were given neocarzinostatin (NCS), a radiomimetic medication that causes DSBs (Ziv et al., 2006). Mouse miRNA microarray analysis was used to determine miRNA expression profile in each sample, which was done at several time points (0–24 hr). As many as 71 distinct miRNAs were found to be considerably (2-fold) upregulated in the NCS-treated Atm+/+ MEFs, but not in the corresponding Atm-/- MEFs, implying that DNA damage stress causes broad-spectrum changes in miRNA expression. According to Wan et al., regulatory RNA-binding proteins in the Drosha and Dicer complexes, such as DDX5 and KSRP, drive posttranscriptional processing of primary and precursor miRNAs after DNA damage. The findings show that nuclear export of pre-miRNAs is increased in an ATM-dependent manner after DNA damage. The ATM-activated AKT kinase phosphorylates Nup153, a main component of the nucleopore, resulting in enhanced interaction between Nup153 and Exportin-5 (XPO5) and increased nuclear export of pre-miRNAs. These findings demonstrate that DNA damage signalling is important for miRNA transport and maturation. In agreement with previous reports showing that ATM-activated p53 and KSRP promote miRNA expression (Suzuki et al., 2009; Zhang et al., 2011), the study found 61 p53-dependent miRNAs and 29 KSRP-dependent miRNAs among the ATM-induced miRNAs.
- In Jiang et al's study, the cellular function and molecular mechanism of miR2045p in hepatocellular cancer were investigated (HCC)(Jiang et al.,2016). Shen et al showed that downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signalling(Shen et al., 2016). A study by Tian et al found that MicroRNA-133b targets Sirt1 and suppresses hepatocellular carcinoma cell progression(Tian et al., 2016). In liver cancer, Yan et al discovered that MicroRNA 486 5p acts as a tumour suppressor of proliferation and cancer stem-like cell characteristics by targeting Sirt1(Yan et al.,2019). Zhang et al reported that MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma (Zhang et al., 2016).
- According to Lu et al, SIRT1 inhibited the growth of gastric cancer through inhibiting the activation of STAT3 and NF-B (Lu et al.,2014). The goal was to look at SIRT1's regulatory effects on gastric cancer (GC) cells (AGS and MKN-45) as well as the links between SIRT1 and STAT3 and NF-B activation in GC cells. The SIRT1 activator (resveratrol RSV) was discovered to contribute to the repression of viability and increase of senescence, which was reversed by SIRT1 inhibitor (nicotinamide NA) and SIRT1 depletion using the CCK-8 and SA-β-gal assays, respectively. SIRT1 activation (RSV supplement) reduced not only STAT3 activation, including STAT3 mRNA level, c-myc mRNA level, phosphorylated STAT3 (pSTAT3) proteins, and acetylizad STAT3 (acSTAT3) proteins, but also pNF-B p65 and acNF-B p65 suppression. The effects of RSV were reversed by NA. Furthermore, when STAT3 or NF-B were knocked down, neither RSV nor NA could affect cellular survival or senescence in MKN-45 cells. Overall, the outcomes of the study revealed that SIRT1 activation could cause GC in vitro to lose viability and senescence. Furthermore, our findings demonstrated that SIRT1 inhibited proliferation in GC cells and was related to deacetylation-mediated suppression of STAT3 and NF-B protein activation. The levels of SIRT1 protein expression in non-small-cell lung cancer (NSCLC) cell lines were examined in a study by Yeung et al.,2004. In comparison to immortalised epithelial human lung NL-20 cells, NSCLC cells exhibit significant quantities of SIRT1 protein, as reported by other researchers (Luo et al. 2001; Vaziri et al, 2001). Pharmacological modulators of Sirtuin activity were employed to see if NF-kB transcription was regulated by Sirtuins (Landry et al, 2000; Bedalov et al, 2001; Howitz et al, 2003). Transient luciferase reporter experiments revealed that cells pretreated with resveratrol had very minimal NF-kB transcription following the presence of TNFα. TNFα-induced NF-kB activity was boosted when cells were pretreated with the Sirtuin inhibitors nicotinamide or spilitomicin. NF-kB transcription was also potentiated in cells treated with trichostatin A (TSA), an HDAC class I and class II inhibitor, as expected.
- In specific subclasses of human breast cancer cells and tumour tissue specimens, an enhanced level of activated NF-kB is found, primarily in erbB2-overexpressing ER-negative breast cancer (Biswas et al 2000;2003). Singh et al explored a variety of methods to inhibit NF-kB activation in ER-negative breast cancer cells and looked at the effects on cell proliferation, apoptosis, and tumour growth in xenografts(Singh et al.,2007). In a prospective cohort study, Sampepajung et al used immunohistochemistry (IHC) to examine NF-B expression and intrinsic subtypes of breast cancer tissue and found a significant correlation between negative ER and overexpression of NF-B (p 0.05), with overexpression of NF-B being higher in negative ER (77.3 percent) compared to positive ER (47.4 percent)(Sampepajung et al., 2021). Laere et al suggested that activation of NF-kB in inflammatory breast cancer (IBC) is associated with loss of estrogen receptor (ER) expression, indicating potential crosstalk between NF-kB and ER(Laere et al.,2007). Differential Sensitivity of ER α and ERβ Cells to the NF-kB Inhibitor Go6976 was tested. A differential sensitivity to Go6976 by ER α and ERβ breast cancer cells was observed (Holloway et al.,2004). The ER α cells were more sensitive and less viable after treatment with this NF-kB inhibitor. The IC50 (50% killing) by Go6976 was 1 mM for Era of MDA-MB435 and MDA-MB231 breast cancer cells, whereas it was greater than 10 mM for ERα of MCF-7 and T47D or the normal mammary epithelial H16N cells. At 10 mM Go6976, about 80% of the ERα cells were killed, whereas only 15–30% of ERα and normal H16N cells were sensitive to this compound. The relative resistance of the H16N normal human mammary cells indicates a possible high therapeutic index of Go6976 against ERα cancer cells.
- Endogenous ER silencing causes EMT in ER-positive breast cancer cells. ER-positive MCF-7 cells were infected with ER shRNA lentiviral particles and stable clones were selected with puromycin (optimal dose of 0.8 g/mL) to knock down ER gene expression (Zheng et al.,2014). When the number of cell passages was increased following infection, the expression of ER was gradually knocked down. Saleh et al. hypothesise that loss of oestrogen receptor function, which causes endocrine resistance in breast cancer, also causes trans-differentiation from an epithelial to a mesenchymal phenotype, which causes enhanced aggressiveness and metastatic tendency(Saleh et al., 2011).
- EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017). Chen et al investigated the potential function of MDM2 in ovarian cancer SKOV3 cells' EMT and metastasis(Chen et al.,2015). TGFβeta and Twist induce EMT by upregulating the expression of EMT markers such Snail, Vimentin, N-cadherin, and ABC transporters like ABCA3, ABCC1, ABCC3, and ABCC10 (Saxena et al., 2011).In the treatment with about 0.3, 3, 30 mM of doxorubicin, human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrate increased cell viability compared to control HMLE, dose-dependently (Saxena et al., 2011).

Weight of Evidence Summary

Increased, DNA damage and mutation leads to Inadequate DNA repair

DNA base excision repair (BER) and, to a lesser extent, nucleotide excision repair (NER) are used to repair oxidative DNA damage. Previous research has found thresholded dose-response curves in oxidative DNA damage and attributed these findings to a lack of repair capability at the curve's inflection point (Gagne et al., 2012; Seager et al., 2012). Following chemical exposures, in vivo, a rise and buildup of oxidative DNA lesions was seen despite the activation of BER, suggesting poor repair of oxidative DNA lesions beyond a certain level(Ma et al., 2008).

Empirical Evidence (include consideration of temporal concordance) has been documented in several studies as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
N,N-dimethylformamide	Homosapiens hepatocyte cell line	In vitro	mM	Increased DNA damage leads to decreased DNA cross link repair mechanisms	Wang et al.,2016
		Experimental			
UV radiation	Cricetulus griseus(Chinese hamster)	-do-	kJ/m2	-do-	Dahle et al.,2008
X-rays	Human leukemia cell line	-do-	Gy/min	-do-	Li et al.,2013
X rays	Mice	In vivo	Gy/min	-do-	Li et al.,2013
		Experimental			
Aniline	Rat	-do-	Kg/day	-do-	Ma et al.,2008

Inadequate DNA repair leads to Increase, Mutations

There will be no increase in mutation frequency if DNA repair is capable of appropriately and efficiently repairing DNA lesions caused by a genotoxic stressor.

For alkylated DNA, for example, efficient AGT removal will result in no increases in mutation frequency. However, once AGT reaches a certain dose, it becomes saturated and can no longer effectively remove alkyl adducts. Mutation occurs when O-alkyl adducts are replicated. The evidence that unrepaired O-alkylated DNA replication induces mutations in somatic cells is vast and has been evaluated. (Basu and Essigmann 1990; Shrivastav et al. 2010).

Empirical Evidence (include consideration of temporal concordance) has been documented in several studies as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
UV radiation	Chinese hamster	In vitro	kJ/m2	inadequate DNA repair	Dahle et al., 2008

UV radiation	Chinese hamster	In vitro	Keratinocytes	Inadequate repair leads to increased mutations	Danie et al.,2006
	Mice	In vivo		-do-	Klungland et al., 1999
X ray	human	In vitro	Gy	-do-(dose-incidence)	Mcmohan et al., 2016

Increase, Mutations leads to Increase,miRNA levels

Evidences suggest that transcription pathway for miRNAs is regulated in the DNA damage response (DDR).Inadequate repair and mutations increase miRNA expression.DNA damage-responsive transcription factors, such as NF-kB, E2F, and Myc, are also involved in miRNA transcription regulation.The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators.The empirical and dose response evidence for increased mutations inducing miRNA expression has been documented as follows;

Compound class	Species	Study type	Dose	KER findings	Reference
Neocarcinostatin	Mouse Fibroblast	In vitro	Ng/ml	Increased mutation leads to increased microRNA expression	Ziv et al.,2006
Neocarcinostatin	Mouse Fibroblast	In vitro	Ng/ml	-do-	Zhang et al.,2011
Cisplatin and IR	Human mammary epithelial cells	In vitro	mM and Gy	-do-	Jaarsveld et al., 2014

Increase,miRNA levels leads to Decrease,SIRT1(sirtuin 1) leves

There are several pathways which suggest suppression of SIRT1 expression when miRNA is elevated.SIRT1 was downregulated at the mRNA and protein levels when miR-138 expression was increased. MiR-138 binds to the SIRT1 gene's 3'UTR unique complimentary site and inhibits SIRT1 expression directly, preventing HCC proliferation, migration, and invasion (Luo et al.,2017).When compared to the normal hepatic cell line L02, SIRT1 is overexpressed, while miR-138 levels are lowered in HepG2, SMMC7721, Bel7404, and HCCM3 .

The evidence for this fact has been listed as follows;

Compound class	Species	Study type	KER findings	Reference
	Human HCC Cell lines	In vitro	Increased miRNA leads to Reduced SIRT1	Jiang et al.,2016; Luo et al.,2017; Tian et al., 2016; Yan et al.,2019; Bae et al.,2014;Zhou et al.,2017
	Human CRC cell lines	In vitro	-do-	Shen et al., 2016;Lian et al.,2018
	Human RCC Cell lines	In vitro	-do-	Zhang et al., 2016;Fu et al.,2018
Astragalus Polysachcharide	Prostate cancer cell lines	In vitro	-do-	Guo et al.,2020;Yang et al.,2014
	Lung cancer cell lines	In vitro	-do-	Guan et al.,2017

Decrease,SIRT1(sirtuin 1) leves leads to Increase activation, Nuclear factor kappa B (NF-kB)

SIRT1 suppresses NF-B signalling either directly by deacetylating the RelA/p65 subunit or indirectly by triggering repressive transcriptional complexes, which frequently involve heterochromatin formation at NF-B promoter regions. SIRT1 expression and signalling are both inhibited by NF-B.

Zhang et al. found that overexpressing RelA/p65 protein increased SIRT1 expression at both the transcriptional and protein levels (36 h treatment), whereas knocking down RelA/p65 expression decreased TNF-induced SIRT1 expression (8 h treatment)(Zhang et al.,2010). They also discovered that the RelA/p65 protein may bind to the SIRT1 promoter's NF-B motifs. These findings suggest that NF-B may promote SIRT1 expression. Given that SIRT1 induction appeared to occur much later than NF-B activation, it appears that this action could represent a feedback response limiting inflammation and eventually generating endotoxin tolerance.

Evidences supporting this key event is as follows;

Compound class	Species	Study type	KER findings	Reference
nicotinamide	Human gastric cancer cell lines	In vitro	Decreased, SIRT1 leads to increased NF kB activity	Lu et al.,2014
nicotinamide or splitomicin	non-small-cell lung cancer (NSCLC) cell lines	In vitro	Decreased, SIRT1 leads to increased NF kB activity	Yeung et al.,2004; Luo et al., 2001; Vaziri et al., 2001

Increase activation, Nuclear factor kappa B (NF-kB) leads to Antagonism, Estrogen receptor

NF-kB activation in breast cancer has been extensively documented in oestrogen receptor negative (ER) breast tumours and ER breast cancer cell lines, implying a significant inhibitory interaction between both signalling pathways (Biswas et al. 2000, 2001, 2004; Zhou et al. 2005). A rise in both NF-kB DNA-binding activity (Nakshatri et al. 1997) and expression of NF-kB target genes such IL8 coincides with a transition from oestrogen dependence to oestrogen independence in breast cancer, indicating inhibitory cross-talk. The fact that some breast tumours that are resistant to the tumoricidal action of anti-estrogens become sensitised to apoptosis and show a drop in NF-kB activity after treatment with oestrogen supports the inverse relationship between ER and NF-kB activity.

-This shows that oestrogen's proapoptotic actions in these tumours are mediated via NF-kB suppression.

Both in vivo and in vitro studies support the finding;

Compound class	Species	Study type	Dose	KER findings	Reference
Bortezomib	Breast cancer cell lines	In vitro		Increased activity of NF- κ B leads to Reduced Estrogen receptor expression	Singh et al., 2017; Holloway et al., 2004
	Human Breast tissue	In vivo		-do-	Biswas et al., 2000; 2003
	Human Breast tissue	In vivo		-do-	Sampepajung et al., 2021; Laere et al., 2007; Indra et al., 2021;

Antagonism, Estrogen receptor leads to EMT

E2/ERa signalling, in part through transcriptional activation of luminal/epithelial-related transcription factors, promotes the development of mammary epithelia along a luminal/epithelial lineage. GATA3 and ERa both promote each other (Eeckhoute et al., 2007). In normal breast epithelia, GATA3 is needed for luminal differentiation (Kouros-Mehr et al., 2008) and GATA3 and ERa control many of the same genes (Wilson et al., 2008). In mice, forcing GATA3 expression in mesenchymal breast cancer cells produces mesenchymal-epithelial transition (MET), a reversible mechanism analogous to EMT, and prevents tumour metastasis (Yan et al., 2010). Another ERa-interacting transcription factor, FOXA1, is essential for luminal lineage in mammary epithelia and stimulates ductal development in mice (Bernardo et al., 2010). FOXA1 enhances ERa gene expression by increasing the accessibility of estrogen-response regions for ERa binding (Nakshatri et al., 2009). In breast cancer cells, on the other hand, E2 appears to increase FOXA1 expression. Importantly, ERa, FOXA1, and GATA3 are all positive breast cancer prognostic factors (Nakshatri et al., 2009).

Ye et al. investigated the impact of ERa overexpression in ERa-negative breast cancer cell lines (MDA-MB-468, MDA-MB-231) or ERa knockdown in ERa-positive cell lines (MCF-7, T47D) on Slug and Snail expression and phenotypes in ERa-positive cell lines (MCF-7, T47D) (Ye et al., 2010). Slug is repressed, E-cadherin is increased, and cells develop as adherent colonies with less invasiveness when ERa is forced to get expressed. ERa knockdown, on the other hand, causes an increase in Slug expression, a decrease in E-cadherin, and spindle-shaped invasive cells.

Wik et al. used integrated molecular profiling to examine Endometrial cancer samples from a primary investigation cohort and three independent validation cohorts (Wik et al., 2013). Patient survival was closely linked to ER- α immunohistochemical staining and receptor gene (ESR1) mRNA expression. In the study cohort, ER- α negative was related with activation of genes implicated in Wnt, Sonic Hedgehog, and TGF- β signalling, indicating epithelial-mesenchymal transition (EMT).

EMT leads to Metastasis, Breast Cancer

The "epithelial-mesenchymal transition" (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas.

EMT is marked by a decrease in E-cadherin and β -catenin expression and an increase in vimentin, fibronectin, and N-cadherin expression (Irani et al., 2018). EMT is a master mechanism in cancer cells that allows them to lose their epithelial characteristics and gain mesenchymal-like qualities. EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017).

Quantitative Consideration

The techniques used for quantifying KE's were reliable with repeatability and reproducibility. Assays were fit for the purpose.

	MIE 1669	KE 155	KE 185	KE1980	KE1981	KE 1172	KE 112	KE1457	AO1982
Human	PCR-RFLP 9-OHdG – ELISA & MDA (Chen CH et al 2011)	-	Acetaldehyde assay, Extract preparation and Western blotting, N ² -Ethyl dGuo quantitation Abraham J et al 2011	-	-	qRT-PCR, immunohistochemistry (McGlynn LM et al 2014)	qRT-PCR, immunohistochemistry (Sampepajung E et al 2021, Van Laere SJ et al 2007.)	IHC, microarray, qPCR, SNP array (Wik et al 2013)	Liang et al., 2013; Liu et al., 2016; Zhang et al., 2015; Chen et al., 2015; Yue et al., 2019; Wang et al., 2018; Yu et al., 2017
Human Tissues	-	-	-	-	qRT-PCR, Western blotting, Luciferase reporter assay H2, H4, H7, H8, H9 Micro-array (Shen ZL et al 2016)	-	-	-	-
Human Cell lines	RT-PCR, Western Blot MAPK assay, Immunoprecipitation, Western immunoblotting Thymidine uptake ECL-SDS PAGE, RIA Adduct removal measurements, DNA isolation, TLC, LCMS Acetaldehyde estimation, DNA adducts – LC-ESI-MS/MS-SRM, Western blotting Western blotting, enzymatic assay,	-	-	Western blotting, clonal survival assay, FACs (van Jaarsveld MT et al 2014)	Micro-array, qRT-PCR, Western blotting, Luciferase reporter assay (Guo S et al 2020, Bae HJ et al 2014, Zhou J et al 2017, Fu H et al 2018, Lian B et al 2018 Guan Y et al 2017 Yang X et al 2014)	qRT-PCR, Luciferase reporter assay Cell based HDAC assay (Yeung F et al 2004)	qPCR, western blotting, immunoprecipitation, immunofluorescent microscopy, Luciferase reporter assay EMSA, IHC, Cell viability assay (Singh S et al 2007, Holloway JN et al 2004, Biswas DK et al 2000, Song RX et al 2005, Scherbakov AM et al 2005, Scherbakov AM et al 2009)	qRT-PCR, cell viability assay, Microarray, wound healing assay, Wound scratch & Transwell assay, Western blotting, EdU incorporation assay (Bours P et al 2015, Liu Y et al 2015, Al Saleh S et al 2011, Zeng Q et al 2014, Ye Y et al 2010, Lin, HY et al 2018)	qRT-PCR, Luciferase reporter assay immunoblotting, immunoprecipitation, cell invasion assay, cell migration assay, bioluminescence imaging, wound healing assay, Wound scratch & Transwell assay, Microarray, Immunofluorescence, Immunohistochemistry, Gujral et al., 2014; Cui et al., 2013; Shiota et al., 2012; Gao et al., 2018; Chen et al., 2017; Liu et al., 2020; Casas et al., 2011; Jackstadt et al., 2013; Kong et al., 2016; Zhang et al., 2014; Huang et al., 2014

	LC-ESI-MS/MS-SRM								
	DNA oxidative damage by ELISA, Immunofluorescence, cell culture, 8-OHdG – ELISA & Ph2AA-focifformation assays, P53 luciferase assays, qPCR, Western Blotting (Eise A. Triano et al 2003, Etique.Nicolas etiqu et al 2004, Izevbigie EB et al 2002, Przyliak A et al 1996, Singletary KW et al 2001, Singletary KW et al 2004, Abraham J et al 2011, Zhao M et al 2017, Jessy Abraham J et al 2011)								
Rat	Free radical assay GC-MS-SIM (Hackney JF et al 1992, McDermott EW et al 1992)	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase. (Kolova N et al, 2013)		Free radicalQuant cell Proliferation assay (Abdelfattah, N. et al 2018)					
Rat Cell lines		Flow cytometric-micronucleus assay, Cell cycle analysis, Replication fork elongation assay, Cytotoxicity assay, Recombination assay, (Panida Navasumrit et al, 2001)							
Mice	Comet assay, ROS generation assay, (Lei Guo et al 2008)	FISH karyotyping, In vivo point mutation assay Garaycoechea JI et al 2018 Whole genome sequencing of HSC clones. (Garaycoechea JI et al, 2012)	In vivo point mutation assay Garaycoechea JI et al 2018	Free radicalQuant cell Proliferation assay (Abdelfattah, N. et al 2018) RNA sequence analysis,Immunostaining,immunoblotting,Flowcytometry,COMET assay,qRT-PCR(Liu Z et al 2017) Microarray (Zhang X et al 2011) qRT-PCR,RIP assay,Immunogold EM(Wan G et al 2013)	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture Bai XZ et al 2018	qRT-PCR,Southern and EMSA,Autoradiography,Immunofluorescent-northern blotting, reporter microscopy, Westernblotting (Biswas DK 2008)		Chen et al.,2017; Gumireddy et al.,2009; Yu et al., 2016; Sarkar et al.,2015	
Canine				micro array(Bukowska M et al 2017)					
Yeast			Fluctuation assay Voordeckers K et al, 2020						

Considerations for Potential Applications of the AOP (optional)

Intended uses of this AOP:

- Helpful for risk assessors, in assessing the risk of alcohol on metastatic breast cancer
- If the causal relationship is established between key events, it may be useful drug targets
- An alternative model to animal model based test methods

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44.Non-Technical Summary Archived 24 July 2006 at the Wayback Machine. UK Committee on Carcinogenicity of Chemicals in Food Consumer Products and the Environment (COC)

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Appendix 1

List of MIEs in this AOP

Event: 1669: Increased, DNA damage and mutation

Short Name: Increased, DNA damage and mutation

Key Event Component

Process	Object	Action
DNA damage response, detection of DNA damage mutation	site of DNA damage	increased
chromosome breakage	chromosome	increased
chromosomal instability	chromosome	increased
abnormal DNA repair	DNA repair complex	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:303 - Frustrated phagocytosis-induced lung cancer	KeyEvent
Aop:409 - Frustrated phagocytosis leads to malignant mesothelioma	KeyEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	KeyEvent
Aop:417 - Aryl hydrocarbon receptor activation leading to lung cancer through AHR-ARNT toxicity pathway	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	MolecularInitiatingEvent
Aop:451 - Interaction with lung resident cell membrane components leads to lung cancer	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals		NCBI

Life Stage Applicability

Life Stage Evidence

Adult

Sex Applicability

Sex Evidence

Unspecific

The DNA damages and mutations can occur in mammals, male or female, and is generally measured in adults.

Key Event Description

DNA damages are alteration of the DNA backbone including abasic site, single or double strand breaks or inter-strand crosslinks. These damages could be recognized and repaired by specialized enzymes. However, if damages persist, mutation in the DNA sequences can occur. Unlike DNA damages, DNA mutations when both strands are modified cannot be repaired and are heritable. Mutations affect the genotype and could affect phenotype.

Different mechanisms are implicated in DNA damage such as oxidative burst, DNA repair dysfunction or centrosome amplification and chromosome instability [1].

How it is Measured or Detected

DNA damages could be measured using different assays, such as micronucleus formation (OECD n°487) [2], comet assay with different protocols for the detection of double and single-strand breaks, DNA-DNA and DNA-protein crosslinks, adduct and oxidized nucleotides (OECD n°489) [3, 4] and γH2AX for the analysis of DNA strand breaks [5].

DNA mutation could be analyzed with Ames test or via the analysis of frequencies of mutations (OECD n°471) [6].

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List of Key Events in the AOP

[Event: 155: Inadequate DNA repair](#)

Short Name: Inadequate DNA repair

Key Event Component

Process	Object	Action
DNA repair	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	KeyEvent
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:322 - Alkylation of DNA leading to reduced sperm count	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	KeyEvent
Aop:432 - Deposition of Energy by Ionizing Radiation leading to Acute Myeloid Leukemia	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	Moderate	NCBI
Syrian golden hamster	Mesocricetus auratus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The retention of adducts has been directly measured in many different types of eukaryotic somatic cells (in vitro and in vivo). In male germ cells, work has been done on hamsters, rats and mice. The accumulation of mutation and changes in mutation spectrum has been measured in mice and human cells in culture. Theoretically, saturation of DNA repair occurs in every species (prokaryotic and eukaryotic). The principles of this work were established in prokaryotic models. Nagel et al. (2014) have produced an assay that directly measures DNA repair in human cells in culture.

NHEJ is primarily used by vertebrate multicellular eukaryotes, but it also been observed in plants. Furthermore, it has recently been discovered that some bacteria (Matthews et al., 2014) and yeast (Emerson et al., 2016) also use NHEJ. In terms of invertebrates, most lack the core DNA-PKcs and Artemis proteins; they accomplish end joining by using the RA50:MRE11:NBS1 complex (Chen et al., 2001). HR occurs naturally in eukaryotes, bacteria, and some viruses (Bhatti et al., 2016).

Key Event Description

DNA lesions may result from the formation of DNA adducts (i.e., covalent modification of DNA by chemicals), or by the action of agents such as radiation that may produce strand breaks or modified nucleotides within the DNA molecule. These DNA lesions are repaired through several mechanistically distinct pathways that can be categorized as follows:

- 1. Damage reversal** acts to reverse the damage without breaking any bonds within the sugar phosphate backbone of the DNA. The most prominent enzymes associated with damage reversal are photolyases (Sancar, 2003) that can repair UV dimers in some organisms, and O6-alkylguanine-DNA alkyltransferase (AGT) (Pegg 2011) and oxidative demethylases (Sundheim et al., 2008), which can repair some types of alkylated bases.
- 2. Excision repair** involves the removal of a damaged nucleotide(s) through cleavage of the sugar phosphate backbone followed by re-synthesis of DNA within the resultant gap. Excision repair of DNA lesions can be mechanistically divided into:
 - a) Base excision repair (BER)** (Dianov and Hübscher, 2013), in which the damaged base is removed by a damage-specific glycosylase prior to incision of the phosphodiester backbone at the resulting abasic site.
 - b) Nucleotide excision repair (NER)** (Schärer, 2013), in which the DNA strand containing the damaged nucleotide is incised at sites several nucleotides 5' and 3' to the site of damage, and a polynucleotide containing the damaged nucleotide is removed prior to DNA resynthesis within the resultant gap.
 - c) Mismatch repair (MMR)** (Li et al., 2016) which does not act on DNA lesions but does recognize mispaired bases resulting from replication errors. In MMR the strand containing the misincorporated base is removed prior to DNA resynthesis.The major pathway that removes oxidative DNA damage is base excision repair (BER), which can be either monofunctional or bifunctional; in mammals, a specific DNA glycosylase (OGG1: 8-Oxoguanine glycosylase) is responsible for excision of 8-oxoguanine (8-oxoG) and other oxidative lesions (Hu et al., 2005; Scott et al., 2014; Whitaker et al., 2017). We note that long-patch BER is used for the repair of clustered oxidative lesions, which uses several enzymes from DNA replication pathways (Klungland and Lindahl, 1997). These pathways are described in detail in various reviews e.g., (Whitaker et al., 2017).
- 3. Single strand break repair (SSBR)** involves different proteins and enzymes depending on the origin of the SSB (e.g., produced as an intermediate in excision repair or due to direct chemical insult) but the same general steps of repair are taken for all SSBs: detection, DNA end processing, synthesis, and ligation (Caldecott, 2014). Poly-ADP-ribose polymerase1 (PARP1) detects and binds unscheduled SSBs (i.e., not deliberately induced during excision repair) and synthesizes PAR as a signal to the downstream factors in repair. PARP1 is not required to initiate SSBR of BER intermediates. The XRCC1 protein complex is then recruited to the site of damage and acts as a scaffold for proteins and enzymes required for repair. Depending on the nature of the damaged termini of the DNA strand, different enzymes are required for end processing to generate the substrates that DNA polymerase β (Polβ; short patch repair) or Pol δ/ε (long patch repair) can bind to synthesize over the gap. Synthesis in long-patch repair displaces a single stranded flap which is excised by flap endonuclease 1 (FEN1). In short-patch repair, the XRCC1/Lig3α complex joins the two ends after synthesis. In long-patch repair, the PCNA/Lig1 complex ligates the ends. (Caldecott, 2014).
- 4. Double strand break repair (DSBR)** is necessary to preserve genomic integrity when breaks occur in both strands of a DNA molecule. There are two major pathways for DSBR: homologous recombination (HR), which operates primarily during S phase in dividing cells, and nonhomologous end joining (NHEJ), which can function in both dividing and non-dividing cells (Teruaki Iyama and David M. Wilson III, 2013).

In higher eukaryotes such as mammals, NHEJ is usually the preferred pathway for DNA DSBR. Its use, however, is dependent on the cell type, the gene locus, and the nuclease platform (Miyaoaka et al., 2016). The use of NHEJ is also dependent on the cell cycle; NHEJ is generally not the pathway of choice when the cell is in the late S or G2 phase of the cell cycle, or in mitotic cells when the sister chromatid is directly adjacent to the double-strand break (DSB) (Lieber et al., 2003). In these cases, the HR pathway is commonly used for repair of DSBs. Despite this, NHEJ is still used more commonly than HR in human cells. Classical NHEJ (C-NHEJ) is the most common NHEJ repair mechanism, but alternative NHEJ (alt-NHEJ) can also occur, especially in the absence of C-NHEJ and HR.

The process of C-NHEJ in humans requires at least seven core proteins: Ku70, Ku86, DNA-dependent protein kinase complex (DNA-PKcs), Artemis, X-ray cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV (Bohaila et al., 2012). When DSBs occur, the Ku proteins, which have a high affinity for DNA ends, will bind to the break site and form a heterodimer. This protects the DNA from exonucleolytic attack and acts to recruit DNA-PKcs, thus forming a trimeric complex on the ends of the DNA strands. The kinase activity of DNA-PKcs is then triggered, causing DNA-PKcs to auto-phosphorylate and thereby lose its kinase activity; the now phosphorylated DNA-PKcs dissociates from the DNA-bound Ku proteins. The free DNA-PKcs phosphorylates Artemis, an enzyme that possesses 5'-3' exonuclease and endonuclease activity in the presence of DNA-PKcs and ATP. Artemis is responsible for 'cleaning up' the ends of the DNA. For 5' overhangs, Artemis nicks the overhang, generally leaving a blunt duplex end. For 3' overhangs, Artemis will often leave a four- or five-nucleotide single stranded overhang (Pardo et al., 2009; Fattah et al., 2010; Lieber et al., 2010). Next, the XLF and XRCC4

proteins form a complex which makes a channel to bind DNA and aligns the ends for efficient ligation via DNA ligase IV (Hammel et al., 2011).

The process of alt-NHEJ is less well understood than C-NHEJ. Alt-NHEJ is known to involve slightly different core proteins than C-NHEJ, but the steps of the pathway are essentially the same between the two processes (reviewed in Chiruvella et al., 2013). It is established, however, that alt-NHEJ is more error-prone in nature than C-NHEJ, which contributes to incorrect DNA repair. Alt-NHEJ is thus considered primarily to be a backup repair mechanism (reviewed in Chiruvella et al., 2013).

In contrast to NHEJ, HR takes advantage of similar or identical DNA sequences to repair DSBs (Sung and Klein, 2006). The initiating step of HR is the creation of a 3' single strand DNA (ss-DNA) overhang. Combinases such as RecA and Rad51 then bind to the ss-DNA overhang, and other accessory factors, including Rad54, help recognize and invade the homologous region on another DNA strand. From there, DNA polymerases are able to elongate the 3' invading single strand and resynthesize the broken DNA strand using the corresponding sequence on the homologous strand.

Fidelity of DNA Repair

Most DNA repair pathways are extremely efficient. However, in principal, all DNA repair pathways can be overwhelmed when the DNA lesion burden exceeds the capacity of a given DNA repair pathway to recognize and remove the lesion. Exceeded repair capacity may lead to toxicity or mutagenesis following DNA damage. Apart from extremely high DNA lesion burden, inadequate repair may arise through several different specific mechanisms. For example, during repair of DNA containing O6-alkylguanine adducts, AGT irreversibly binds a single O6-alkylguanine lesion and as a result is inactivated (this is termed suicide inactivation, as its own action causes it to become inactivated). Thus, the capacity of AGT to carry out alkylolation repair can become rapidly saturated when the DNA repair rate exceeds the de novo synthesis of AGT (Pegg, 2011).

A second mechanism relates to cell specific differences in the cellular levels or activity of some DNA repair proteins. For example, XPA is an essential component of the NER complex. The level of XPA that is active in NER is low in the testes, which may reduce the efficiency of NER in testes as compared to other tissues (Köberle et al., 1999). Likewise, both NER and BER have been reported to be deficient in cells lacking functional p53 (Adimoolam and Ford, 2003; Hanawalt et al., 2003; Seo and Jung, 2004). A third mechanism relates to the importance of the DNA sequence context of a lesion in its recognition by DNA repair enzymes. For example, 8-oxoguanine (8-oxoG) is repaired primarily by BER; the lesion is initially acted upon by a bifunctional glycosylase, OGG1, which carries out the initial damage recognition and excision steps of 8-oxoG repair. However, the rate of excision of 8-oxoG is modulated strongly by both chromatin components (Menoni et al., 2012) and DNA sequence context (Allgayer et al., 2013) leading to significant differences in the repair of lesions situated in different chromosomal locations.

DNA repair is also remarkably error-free. However, misrepair can arise during repair under some circumstances. DSB is notably error prone, particularly when breaks are processed through NHEJ, during which partial loss of genome information is common at the site of the double strand break (Iyama and Wilson, 2013). This is because NHEJ rejoins broken DNA ends without the use of extensive homology; instead, it uses the microhomology present between the two ends of the DNA strand break to ligate the strand back into one. When the overhangs are not compatible, however, indels (insertion or deletion events), duplications, translocations, and inversions in the DNA can occur. These changes in the DNA may lead to significant issues within the cell, including alterations in the gene determinants for cellular fatality (Moore et al., 1996).

Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including, Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under s conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).

Misrepair may also occur through other repair pathways. Excision repair pathways require the resynthesis of DNA and rare DNA polymerase errors during gap resynthesis will result in mutations (Brown et al., 2011). Errors may also arise during gap resynthesis when the strand that is being used as a template for DNA synthesis contains DNA lesions (Kozmin and Jinks-Robertson, 2013). In addition, it has been shown that sequences that contain tandemly repeated sequences, such as CAG triplet repeats, are subject to expansion during gap resynthesis that occurs during BER of 8-oxoG damage (Liu et al., 2009).

How it is Measured or Detected

There is no test guideline for this event. The event is usually inferred from measuring the retention of DNA adducts or the creation of mutations as a measure of lack of repair or incorrect repair. These 'indirect' measures of its occurrence are crucial to determining the mechanisms of genotoxic chemicals and for regulatory applications (i.e., determining the best approach for deriving a point of departure). More recently, a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) has been developed to directly measure the ability of human cells to repair plasmid reporters (Nagel et al., 2014).

Indirect Measurement

In somatic and spermatogenic cells, measurement of DNA repair is usually inferred by measuring DNA adduct formation/removal. Insufficient repair is inferred from the retention of adducts and from increasing adduct formation with dose. Insufficient DNA repair is also measured by the formation of increased numbers of mutations and alterations in mutation spectrum. The methods will be specific to the type of DNA adduct that is under study.

Some EXAMPLES are given below for alkylated DNA.

DOSE-RESPONSE CURVE FOR ALKYL ADDUCTS/MUTATIONS: It is important to consider that some adducts are not mutagenic at all because they are very effectively repaired. Others are effectively repaired, but if these repair processes become overwhelmed mutations begin to occur. The relationship between exposure to mutagenic agents and the presence of adducts (determined as adducts per nucleotide) provide an indication of whether the removal of adducts occurs, and whether it is more efficient at low doses. A sub-linear DNA adduct curve suggests that less effective repair occurs at higher doses (i.e., repair processes are becoming saturated). A sub-linear shape for the dose-response curves for mutation induction is also suggestive of repair of adducts at low doses, followed by saturation of repair at higher doses. Measurement of a clear point of inflection in the dose-response curve for mutations suggests that repair does occur, at least to some extent, but reduced repair efficiency arises above the breakpoint. A lack of increase in mutation frequencies (i.e., flat line for dose-response) for a compound showing a dose-dependent increase in adducts would imply that the adducts formed are either not mutagenic or are effectively repaired.

RETENTION OF ALKYL ADDUCTS: Alkylated DNA can be found in cells long after exposure has occurred. This indicates that repair has not effectively removed the adducts. For example, DNA adducts have been measured in hamster and rat spermatogonia several days following exposure to alkylating agents, indicating lack of repair (Seiler et al., 1997; Scherer et al., 1987).

MUTATION SPECTRUM: Shifts in mutation spectrum (i.e., the specific changes in the DNA sequence) following a chemical exposure (relative to non-exposed mutation spectrum) indicates that repair was not operating effectively to remove specific types of lesions. The shift in mutation spectrum is indicative of the types of DNA lesions (target nucleotides and DNA sequence context) that were not repaired. For example, if a greater proportion of mutations occur at guanine nucleotides in exposed cells, it can be assumed that the chemical causes DNA adducts on guanine that are not effectively repaired.

Direct Measurement

Nagel et al. (2014) we developed a fluorescence-based multiplex flow-cytometric host cell reactivation assay (FM-HCR) to measures the ability of human cells to repair plasmid reporters. These reporters contain different types and amounts of DNA damage and can be used to measure repair through by NER, MMR, BER, NHEJ, HR and MGMT.

Please refer to the table below for additional details and methodologies for detecting DNA damage and repair.

Assay Name	References	Description	DNA Damage/Repair Being Measured	OECD Approved Assay
Dose-Response Curve for Alkyl Adducts/Mutations	Lutz 1991 Clewell 2016	Creation of a curve plotting the stressor dose and the abundance of adducts/mutations; Characteristics of the resulting curve can provide information on the efficiency of DNA repair	Alkylation, oxidative damage, or DSBs	N/A
Retention of Alkyl Adducts	Seiler 1997 Scherer 1987	Examination of DNA for alkylation after exposure to an alkylating agent; Presence of alkylation suggests a lack of repair	Alkylation	N/A
		Shifts in the mutation spectrum after	Alkylation.	

Mutation Spectrum	Wyrick 2015	exposure to a chemical/mutagen relative to an unexposed subject can provide an indication of DNA repair efficiency, and can inform as to the type of DNA lesions present	oxidative damage, or DSBs	N/A
DSB Repair Assay (Reporter constructs)	Mao et al., 2011	Transfection of a GFP reporter construct (and DsRed control) where the GFP signal is only detected if the DSB is repaired; GFP signal is quantified using fluorescence microscopy or flow cytometry	DSBs	N/A
Primary Rat Hepatocyte DNA Repair Assay	Jeffrey and Williams, 2000 – Butterworth et al., 1987	Rat primary hepatocytes are cultured with a ³ H-thymidine solution in order to measure DNA synthesis in response to a stressor in non-replicating cells; Autoradiography is used to measure the amount of ³ H incorporated in the DNA post-repair	Unscheduled DNA synthesis in response to DNA damage	N/A
Repair synthesis measurement by ³ H-thymine incorporation	Iyama and Wilson, 2013	Measure DNA synthesis in non-dividing cells as indication of gap filling during excision repair	Excision repair	N/A
Comet Assay with Time-Course	Olive et al., 1990 – Trucco et al., 1998	Comet assay is performed with a time-course; Quantity of DNA in the tail should decrease as DNA repair progresses	DSBs	Yes (No, 489)
Pulsed Field Gel Electrophoresis (PFGE) with Time-Course	Biedermann et al., 1991	PFGE assay with a time-course; Quantity of small DNA fragments should decrease as DNA repair progresses	DSBs	N/A
Fluorescence - Based Multiplex Flow-Cytometric Host Reactivation Assay (FM-HCR)	Nagel et al., 2014	Measures the ability of human cells to repair plasma reporters, which contain different types and amounts of DNA damage; Used to measure repair processes including HR, NHEJ, BER, NER, MMR, and MGMT	HR, NHEJ, BER, NER, MMR, or MGMT	N/A

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Event: 185: Increase, Mutations

Short Name: Increase, Mutations

Key Event Component

Process	Object	Action
mutation	deoxyribonucleic acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:15 - Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	AdverseOutcome
Aop:293 - Increased DNA damage leading to increased risk of breast cancer	AdverseOutcome
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations	AdverseOutcome
Aop:272 - Deposition of energy leading to lung cancer	KeyEvent
Aop:397 - Bulky DNA adducts leading to mutations	AdverseOutcome
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name

Assorted Gene Loci Mutation Assays	Tindall et al., 1989; Kruger et al., 2015	After exposure to a chemical/mutagen, mutations can be measured by the ability of exposed cells to form colonies in the presence of specific compounds that would normally inhibit colony growth; Usually only cells -/- for the gene of interest are able to form colonies	N/A
TK Mutation Assay	Yamamoto et al., 2017; Liber et al., 1982; Lloyd and Kidd, 2012	After exposure to a chemical/mutagen, mutations are detected at the thymidine kinase (TK) loci of L5178Y wild-type mouse lymphoma TK (+/-) cells by measuring resistance to lethaltrifluorothymidine (TFT); Only TK-/- cells are able to form colonies	Yes (No. 490)
HPRT Mutation Assay	Ayres et al., 2006; Parry and Parry, 2012	Similar to TK Mutation Assay above, X-linked HPRT mutations produced in response to chemical/mutagen exposure can be measured through colony formation in the presence of 6-TG or 8-azoguanine; Only HPRT-/- cells are able to form colonies	Yes (No. 476)
Salmonella Mutagenicity Test (Ames Test)	OECD, 1997	After exposure to a chemical/mutagen, point mutations are detected by analyzing the growth capacity of different bacterial strains in the presence and absence of various metabolic activation systems	Yes (No. 471)
PIG-A / PIG-O Assay	Kruger et al., 2015; Nakamura, 2012; Chikura, 2019	After exposure to a chemical/mutagen, mutations in PIG-A or PIG-O (which decrease the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor protein) are assessed by the colony-forming capabilities of cells after <i>in vitro</i> exposure, or by flow cytometry of blood samples after <i>in vivo</i> exposure	N/A
Single Molecule PCR	Kraytsberg, 2005; Yauk, 2002	This PCR technique uses a single DNA template, and is often employed for detection of mutations in microsatellites, recombination studies, and generation of colonies	N/A
ACB-PCR	Myers et al., 2014 (Textbook, pg 345-363); Banda et al., 2013; Banda et al., 2015; Parsons et al., 2017	Using this PCR technique, single base pair substitution mutations within oncogenes or tumour suppressor genes can be detected by selectively amplifying specific point mutations within an allele and selectively blocking amplification of the wild-type allele	N/A
Transgenic Rodent Mutation Assay	OECD 2013; Lambert 2005; Lambert 2009	This <i>in vivo</i> test detects gene mutations using transgenic rodents that possess transgenes and reporter genes; After <i>in vivo</i> exposure to a chemical/mutagen, the transgenes are analyzed by transfecting bacteria with the reporter gene and examining the resulting phenotype	Yes (No. 488)
Conditionally inducible transgenic mouse models	Parsons 2018 (Review)	Inducible mutations linked to fluorescent tags are introduced into transgenic mice; Upon exposure of the transgenic mice to an inducing agent, the presence and functional assessment of the mutations can be easily ascertained due to expression of the linked fluorescent tags	N/A
Error-Corrected Next Generation Sequencing (NGS)	Salk 2018 (Review)	This technique detects rare subclonal mutations within a pool of heterogeneous DNA samples through the application of new error-correction strategies to NGS; At present, few laboratories in the world are capable of doing this, but commercial services are becoming available (e.g., Duplex sequencing at TwinStrand BioSciences)	N/A

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Event: 1554: Increase Chromosomal Aberrations

Short Name: Increase chromosomal aberrations

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage Evidence

All life stages High

Sex Applicability

Sex Evidence

Unspecific High

Chromosomal aberrations indicating clastogenicity can occur in any eukaryotic or prokaryotic cell. However, dose-response curves can differ depending on the cell cycle stage when the DSB agent was introduced (Obe et al., 2002).

Key Event Description

The term "structural chromosomal aberrations" refers to chromosome damage caused by breaks in the DNA that can result in the deletion, addition, or rearrangement of chromosomal segments. According to whether one or both chromatids are affected, chromosomal aberrations can be classified into two main groups: chromatid-type and chromosome-type. Additionally, they can be divided into rejoined and non-rejoined aberrations. Translocations, insertions, dicentric, and rings are examples of rejoined aberrations, whereas acentric fragments and breaks are examples of unrejoined aberrations (Savage, 1976). Some of these abnormalities, like reciprocal translocations, are long-lasting and can last for many years (Tucker and Preston, 1996). Others, such as dicentric and acentric fragments, are unstable and weaken with each cell division due to cell death (Boei et al., 1996). After cell division, these activities might still be visible, and the DNA is irreversibly damaged. The occurrence of chromosomal abnormalities is linked to cancer development and cell death (Mitelman, 1982).

A missing, excess, or asymmetrical part of chromosomal DNA is referred to as a chromosomal aberration (CA). There are various double-strand break (DSB) repair mechanisms that could be responsible for these DNA modifications in the chromosome structure (Obe et al., 2002).

The four basic categories of CAs are inversions, translocations, duplications, and deletions. When a section of a chromosome's genetic material is destroyed, deletions take place. When a chromosome's end portion is cut, terminal deletions result.

When a chromosome splits into two different places and wrongly rejoins, leaving the middle portion out, interstitial deletions result. Duplications occur when excess genetic material is added to or rearranged; they can take the forms of transpositions, tandem duplications, reverse duplications, and misplaced duplications (Griffiths et al., 2000). A segment of one chromosome is transferred to a non-homologous chromosome in translocations (Bunting and Nussenzweig, 2013). A reciprocal translocation occurs when regions of two non-homologous chromosomes are switched. When an inversion occurs, the DNA sequence is effectively reversed because both ends of the chromosome split and are ligated at the opposite ends.

The copy number variant is a fifth type of CA that can exist in the genome (CNV). CNVs are deletions or duplications that can range in size from 50 base pairs (Arlt et al., 2012; Arlt et al., 2014; Liu et al., 2013) up into the megabase pair range and may make up more than 10% of the human genome (Shlien et al., 2009; Zhang et al., 2016; Hastings et al., 2009). (Arlt et al., 2012; Wilson et al., 2015; Arlt et al., 2014; Zhang et al., 2016). According to Wilson et al. (2015), CNV regions are particularly abundant in large active transcription units and genes, and they are especially problematic when they result in the duplication of oncogenes or the loss of tumor suppressor genes (Liu et al., 2013; Curtis et al., 2012).

Recurrent and non-recurrent CNVs are two different types. Non-allelic homologous recombination (NAHR), a recombination process that occurs during meiosis, is hypothesised to be the cause of recurrent CNVs (Arlt et al., 2012; Hastings et al., 2009). These germline CNVs, also known as recurrent CNVs, may be inherited and are hence prevalent in various people (Shlien et al., 2009; Liu et al., 2013). It is thought that non-recurrent CNVs are created in mitotic cells during the replication process. It has been proposed that replication-related stress, particularly stalled replication forks, triggers microhomology-mediated processes to break the replication stall, which frequently leads to duplications or deletions, despite the fact that the mechanism is not well understood.

Two models that have been proposed to explain this mechanism include the Fork Stalling and Template Switching (FoSTeS) model, and the Microhomology-Mediated Break-Induced Replication (MMBIR) model (Arlt et al., 2012; Wilson et al., 2015; Lee et al., 2007; Hastings et al., 2009).

Depending on whether the aberration affects the chromatid or the chromosome, CAs can be categorized. Chromosome-type aberrations (CSAs) are chromosome breakage and chromatid swaps; ring chromosomes, marker chromosomes, and dicentric chromosomes are examples of chromatid-type aberrations (CTAs) (Bonassi et al., 2008; Hagmar et al., 2004). Micronuclei (MN; small nucleus-like structures that contain a chromosome or a fragment of a chromosome that was lost during mitosis) and nucleoplasmic bridges (NPBs; physical linkages between the two nuclei) are visible in binucleated cells when cells are halted at the cytokinesis step (El-Zein et al., 2014). The DNA sequence can be examined to evaluate other CAs, as it is for identifying copy number variants (CNVs) (Liu et al., 2013).

How it is Measured or Detected

Assay	References	Description
Fluorescent In Situ Hybridization (FISH)	Beaton et al., 2013; Pathak et al., 2017	Fluorescent assay of metaphase chromosomes that can detect CAs through chromosome painting and microscopic analysis
Cytokinesis Block Micronucleus (CBMN) Assay with Microscopy in vitro	Fenech, 2000; OECD, 2016a	Cells are cultured with cytokinesis blocking agent, fixed to slides, and undergo MN quantification using microscopy.
Micronucleus (MN) Assay by Microscopy in vivo	OECD, 2016b	Cells are fixed on slides and MN are scored using microscopy. Red blood cells can also be scored for MN using flow cytometry (see below)
CBMN with Imaging Flow Cytometry	Rodrigues et al., 2015	Cells are cultured with cytokinesis blocking agent, fixed in solution, and imaged with flow cytometry to quantify MN
Flow cytometry detection of MN	Dertinger et al., 2004; Bryce et al., 2007; OECD 2016a, 2016b	In vivo and in vitro flow cytometry-based, automated micronuclei measurements are also done without cytokinesis block. MN analysis in vivo is performed in peripheral blood cells to detect MN in erythrocytes and reticulocytes.
High-throughput biomarker assays (indirect measures to	Bryce et al. 2014, 2016, 2018 Khouri et al., 2013, Khouri et al., 2016)	Multiplexed biomarkers can be measured by flow cytometry are used to discern clastogenic and aneugenic mechanisms for MN induction. Flow cytometry-based quantification of γH2AX foci and p53 protein expression (Bryce et al., 2016). Prediscreen Assay– In-Cell

confirm clastogenicity)	Hendriks et al., 2012, 2016; Wink et al., 2014	Western-based quantification of γH2AX Green fluorescent protein reporter assay to detect the activation of stress signaling pathways, including DNA damage signaling including a reporter porter that is associated with DNA double strand breaks.
Dicentric Chromosome Assay (DCA)	Abe et al., 2018	Cells are fixed on microscope slides, chromosomes are stained, and the number of dicentric chromosomes are quantified
High content imaging	Shahane et al., 2016	DNA can be stained using fluorescent dyes and micronuclei can be scored high-throughput microscopy image analysis.
Chromosomal aberration test	OECD, 2016c; 2016d; 2016e	In vitro, the cell cycle is arrested at metaphase after 1.5 cell cycle following 3-6 hour exposure In vivo, the test chemical is administered as a single treatment, bone marrow is collected 18-24 hrs later (TG 475), while testis is collected 24-48 hrs later (TG 483). The cell cycle is arrested with a metaphase-arresting chemical (e.g., colchicine) 2-5 hours before cell collection. Once cells are fixed and stained on microscope slides, chromosomal aberrations are scored
Array Comparative Genomic Hybridization (aCGH) or SNP Microarray	Adewoye et al., 2015; Wilson et al., 2015; Arlt et al., 2014; Redon et al., 2006; Keren, 2014; Mukherjee, 2017	CNVs are most commonly detected using global DNA microarray technologies; This method, however, is unable to detect balanced CAs, such as inversions
Next Generation Sequencing (NGS): Whole Genome Sequencing (WGS) or Whole Exome Sequencing (WES)	Liu, 2013; Shen, 2016; Mukherjee, 2017	CNVs are detected by fragmenting the genome and using NGS to sequence either the entire genome (WGS), or only the exome (WES); Challenges with this methodology include only being able to detect CNVs in exon-rich areas if using WES, the computational investment required for the storage and analysis of these large datasets, and the lack of computational algorithms available for effectively detecting somatic CNVs

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Event: 1980: Increased micro RNA expression

Short Name: Increase,miRNA levels

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability		Evidence	Links
Term	Scientific Term		
mice	Mus sp.	Moderate	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	Moderate

Regulation of miRNA expression by DNA replication,damage and repair responses,transcription and translation has been proved in animals like mice,canine and cell line experiments.

Key Event Description

Biological state

Genome integrity must be maintained for the proper functioning and survival of an organism. There has been an efficient and rapid response developed by the eukaryotic cells to DNA damage to overcome the harmful effects. As soon as the DNA damage or replication arrest is detected, the activation of cell cycle checkpoint and stopping the progress of the cell cycle thus providing time for the cell to repair the DNA damage. The response to DNA damage also leads to transcriptional regulation, activation of DNA repair, and, in severe cases, initiation of apoptosis (Harper, J.W., and Elledge, S.J. , 2007). Expression of miRNAs may be regulated by the DNA damage response. A study reported that that micro RNA expression is a partially ATM / ATR-independent manner(Pothof, J. et al , 2009). Subsequent studies have shown that the tumor suppressor p53 promotes PrimeRNA processing via RNA helicase p68 (Suzuki, H.I et al, 2009).

Han et al evaluated miRNA expression pattern in a DNA damage regulatory protein, DDX1 in controls, as well in DDX1-knockdown U2OS cells with the help of reverse transcription quantitative-PCR (qRT-PCR) and human miRNA array (Han C et al, 2014). The study noticed a significant reduction in the expression levels of a subset of miRNAs -200 family such as miR-200a, miR-200b, miR-200c, miR-141 and miR-429 (cut-off >2-fold),d miR-429). The ovarian

cancer genomics study revealed a 8-miRNA signature that defines the mesenchymal subtype of serous ovarian cancer (Yang Y, et al, 2011). Among the eight miRNAs, miR-200a, miR-29c, miR-141 and miR-101 were significantly dependent on DDX1, suggesting that DDX1 may play a role in ovarian tumor progression. Nuclear run-on assays were performed to determine whether DDX1 regulates the miRNA expression at transcriptional or post-transcriptional levels. No notable differences were seen in the transcription of pri-miR-200s from the two miR-200 gene clusters (miR-200a/200b/429 and miR-200c/141) in the control and DDX1-silenced cells . However, in the DDX1-knockdown U2OS cells, the levels of mature DDX1-dependent miRNAs, but not control miR-21, were significantly decreased. Due to the potential inhibition of miRNA processing activity, primary transcripts of the DDX1-dependent miRNAs were accumulated. Conversely, these DDX1-dependent miRNAs were up-regulated in the DDX1- overexpressing cells. The above findings suggested that expression of specific miRNAs was promoted by DDX1 at the post-transcriptional level.

Biological compartments:

Cellular, nucleus, cytoplasm and mitochondria

General role in biology:

MicroRNAs (miRNAs) are endogenous non-coding RNAs that contain approximately 22 nucleotides. They function as major regulators of various biological processes, and their dysregulation is associated with many diseases, including cancer.

Cells trigger a specific cellular responses to preserve the integrity of the genome. The DNA damage response (DDR) is one among them along with several distinct DNA repair pathways. Normal cells need to repair DNA damage through various repair mechanisms or induce apoptosis and cell cycle arrest if repair is not possible [Jackson SP and Bartek J, 2009]. Genomic instability and mutagenesis are brought about by the disruption of repair mechanisms. DNA damage response (DDR) determines the fate of the cell and controls microRNAs expression. This will in turn regulate important components of the DNA repair machinery. Various reports suggest the key role of miRNA in the regulation of the DDR [d'Adda di Fagnaga F, 2014 and WeiW et al 2012]. The DDR and DNA damage are known regulators of miRNA expression [Sharma V et al 2013 and Chowdhury D et al 2013]. Several studies have shown that the cellular sensitivity to chemotherapeutic drugs is affected by DDR- miRNA network [van Jaarsveld MT et al 2014].

A bidirectional relationship between miRNAs and the DDR has been suggested by studies. The DDR is a known regulator of miRNA expression at both transcriptional and post-transcriptional levels, and miRNA-mediated gene silencing has been shown to modulate the activity of the DDR [d'Adda di Fagnaga F, 2014 ; WeiW et al 2012 and Han C et al 2012]. A unique set of miRNAs as well as a common core miRNA signature are activated depending on DNA damage type and level, suggesting that miRNAs regulate the DDR by mechanisms based on the type and/or the intensity of DNA damage [Han C et al 2012]. miRNAs expression may be regulated by transcription factors either binding directly to miRNA promoters and modulating their transcriptional activity, or by modifying the expression of miRNA processing machinery components.

Studies have widely explored the TP53-mediated transcriptional pathways regulating miRNA expression following DNA damage. miRNA-34a-c is induced by DNA damage and oncogenic stress, is one of the transcriptional target of the tumor suppressor TP53 [Hermeking H et al 2012]. TP53 directly binds to the promoter of miRNA-34 and activates transcription. Micro RNA-34 has been reported to repress the mRNA transcripts of several genes involved in the regulation of cell cycle, cell proliferation and survival, such as BCL2, CCND1, CNE2, MYC, CDK4, CDK6 and SIRT1 [Hermeking H et al 2012]. Activation of miRNA-34a promotes TP53-mediated apoptosis, cell cycle arrest or senescence [Hermeking H et al 2012]. miRNA-34a may target SIRT1, form a positive feedback loop of the acetylation of TP53, expression of its transcriptional targets, regulating cell cycle and apoptosis [Hermeking H et al 2012].

The alternative pathway involving p38 MAPK signaling also induces miR-34c [Cannell IG et al 2010]. Inhibition of miRNA 34 prevents the DNA damage induced cell cycle arrest and results in an increased DNA synthesis [Cannell IG et al 2010].

DNA damage promotes the TP53-dependent upregulation of miRNA-192, miRNA-194 and miRNA-215. The genomic region surrounding the miRNA-194/miRNA-215 cluster contains a putative TP53-binding element, indicating that these miRNAs are transcriptionally activated by TP53 [Hermeking H et al 2012]. The expression of miRNA-192 and miRNA-215 induces cell cycle arrest and targets several transcripts involved in cell cycle checkpoints [Georges SA et al 2008].

MYC and E2F, are the two other transcription factors involved in DNA damage- induced cell cycle checkpoints, that regulate the expression of several miRNAs. Both factors induce transcription of the miRNA-17-92 cluster that forms a feedback loop by inhibiting E2F expression [Aguda BD et al 2008]. E2F transcription factors are repressed by several other miRNAs, including miRNA-106a-92 and miRNA-106b-25 cluster members, miRNA-210, miRNA-128, miRNA-34 and miRNA-20 [Wan G et al 2011].

DNA damage upregulates several miRNAs, including miRNA-16-1, miRNA-143 and miRNA-145. [Suzuki HI et al 2009]. Most TP53 mutations found in cancers are located in a domain required for miRNA processing and transcriptional activity [Suzuki HI et al 2009]. Thus, loss of TP53 functions in miRNAs transcription and processing might contribute to cancer progression. Considering that some miRNAs are reduced after DNA damage in an ATM-dependent manner, ATM could be also involved in inhibitory pathways that downregulate miRNA expression [Wang Y et al 2013]. These findings support the existence of a critical link between the DDR and miRNA processing pathway.

In the DNA damage response, post-transcriptional processing of miRNAs is also regulated. It was reported that DNA damage led to increased levels of some pre-miRNAs and mature miRNAs without significant changes of levels of their primary transcripts, suggesting posttranscriptional mechanisms could contribute to the induction of certain miRNAs under DNA damage stress [Zhang X, et al 2011]. There appears to be functional connections between DNA damage response and miRNA processing and maturation.

Micro RNA - 18a, miR-100, miR-101, miR-181, and miR-421, have been implicated as novel regulators to control the protein level of ATM [Majid S et al 2010]. BRCA1, a critical tumor suppressor, BRCA1, is also recruited to DNA damage lesions, where it facilitates DNA repair. The level of BRCA1 is regulated by miR-182, miR-146a, and 146b-5p [Matsui M et al 2013].

The tumor suppressor p53 has a central role in the activation of genes in multiple pathways, including cell cycle regulation, tumor suppression, and apoptosis. Micro RNA-125b and miR-504 have been identified as negative regulators of p53 in several types of human cells [Kreis S et al 2008 and Wang J et al 2012].

The available evidence suggests that DNA damage signaling participates in miRNA biogenesis by regulating both transcriptional and post-transcriptional mechanisms. Further studies can through light on the correlation between DNA damaging signaling and miRNA processing. The majority of the studies have examined the miRNA regulation in response to DNA damage and have focused on events that occur in the nucleus. It is important to extend the investigations in understanding the contribution of cytoplasmic regulation of miRNA biogenesis following DNA damage. It is very interesting to determine whether DNA damage signals can modulate the turnover, stabilization, modification, and degradation of miRNAs.

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	Western blotting, clonal survival assay, FACS (van Jaarsveld MT et al 2014)	Yes	Strong	Yes	Yes	Yes
Mice	Free radicCyQuant cell Proliferation assay (Abdelfattah, N. et al 2018)	Yes	Strong	Yes	Yes	Yes
	RNA sequence analysis, Immunostaining, immunoblotting, Flow cytometry, COMET assay, qRT PCR (Liu Z et al 2017)	Yes	Strong	Yes	Yes	Yes
	Microarray (Zhang X et al 2011)	Yes	Strong	Yes	Yes	Yes
	qRT PCR, RIP assay, Immunogold EM (Wan G et al 2013)	Yes	Strong	Yes	Yes	Yes
Canine	micro array (Bulkowska M et al 2017)	Yes	Strong	Yes	Yes	Yes

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Zhang, X., Wan, G., Berger, F. G., He, X., & Lu, X. (2011). The ATM kinase induces microRNA biogenesis in the DNA damage response. *Molecular cell*, 41(4), 371-383.

Event: 1981: Decreased SIRT1 expression

Short Name: Decrease,SIRT1(sirtuin 1) levels

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability			
Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability	
Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability	
Sex	Evidence
Female	Moderate

Decreased SIRT1 expression is known to be highly conserved throughout evolution and is present from humans to invertebrates.

Key Event Description

Biological state:

Mammalian SIRT5 include seven proteins (SIRT1-7) with deacetylase activity belonging to the class III histone deacetylase family. SIRT5 share homology with the yeast deacetylase Sir2, and have different sequences and lengths in both their N- and C-terminal domains (Carafa, V. et al 2012). Expressed from bacteria to humans (Vaquero, A. 2009), SIRT5 target histone and non-histone proteins.

Localization of SIRT5 is restricted to mitochondria, cytoplasm and nucleus The location of SIRT1, SIRT6, and SIRT7 is predominantly in the nucleus, while SIRT2 in the cytosol, and SIRT3, SIRT4, and SIRT5 are in the mitochondria. Depending on their role in regulating different pathways, SIRT5 relocate under different conditions such as cell cycle phase, tissue type, developmental stage, stress condition, and metabolic status which has been documented in the literature. (McGuinness, D. et al 2011). As per Mitchishita et al, SIRT1, SIRT2, and SIRT7 are often found in both the nucleus and cytoplasm (Michishita, E et al 2005).

Cellular pathways like DNA repair, transcriptional regulation, metabolism, aging, and senescence are modulated by Sirtuins. This has created sufficient interest with Sirtuins as target in cancer research as the above mentioned functions are involved in initiation and progression of cancer. Evidence have suggested the association of SIRT's with metabolism-associated TFs, MYC and hypoxia inducible factor-1 (HIF-1), in terms of energy metabolic reprogramming.(Zwaans, B. M. et al 2014)

The biological effect of SIRT's in cancer is either tumor suppression or tumor promoter (oncogenes) action by altering the cell proliferation, differentiation, and death which in turn depends on cell context and experimental conditions. These two totally opposite function of SIRT's on cancer cell is remains a highly debated and controversial topic. Whether SIRT's act as tumor suppressors or promoters depends on (i) their The different expression levels of SIRT's in tumors and its effects on cell cycle, cell growth, death, their action on specific proto-oncogene and onco-suppressor proteins will determine SIRT's role as tumor suppressor or tumor promoters (Deng, C. X. 2009).

Sirtuin Reactions

The NAD⁺-dependent deacetylation is well known enzymatic reaction catalyzed by SIRT's. Deacetylation reaction begins with amide cleavage from NAD⁺ with the formation of nicotinamide and an intermediate of reaction, O-ADP-ribose. This intermediate formed is necessary for the deacetylation process by which SIRT's catalyze the transfer of one acetyl group from a lysine to O-ADP-ribose moiety to form O-acetyl-ADP-ribose and the deacetylated lysine product. This reaction requires a mole equivalent of NAD⁺ per acetyl group removed and is controlled by the cellular [NAD]/[NADH] ratio (Sauve, A. A. 2010 and Shi, Y. et al 2013).

Among the SIRT's family, only SIRT1, SIRT2, and SIRT3 possess a robust deacetylase activity even though SIRT enzymes are primarily known as protein deacetylases. SIRT4, SIRT5, SIRT6, and SIRT7) exhibit a weak or no detectable deacetylation activity at all.Through these reactions, SIRT's are able to regulate several key cellular processes (Jiang, H., et al 2013 and Zhang, S. et al 2017).

Biological compartments:

Regulation of gene expression takes place in the cell, subcellular site being nucleus.

General role in biology:

Silent Inflammation Regulator 2 (SIR2) proteins belong to the family of histone deacetylases (HDACs) that catalyze deacetylation of both histone and non- histone lysine residues.

Mammalian sirtuins (SIRT1-7) are involved in diverse biological processes including energy metabolism, lifespan and health span regulation (Longo VD et al 2006). Mammalian sirtuins possess will bring about an array of biological functions through its enzymatic activity such as histone deacetylase, mono-ADP-ribosyltransferase, desuccinylase, demalonylase, demyristoylase, and depalmitoylase activity (Michan S et al 2007). SIRT1 located in the nucleus play an important role in genomic stability, telomere maintenance, and cell survival (Chen J et al 2011 and, Haigis MC et al 2006).

Among the 7 SIRT's, SIRT1 is the largest in terms of total DNA and amino acid sequence studied sirtuin [Fang, Y. and M.B. Nicholl 2011]. SIRT1, a class 3 histone deacetylase, is implicated in the modulation of apoptosis, senescence, proliferation, and aging. It's actions arebrought about by cellular nicotinamide adenosine dinucleotide (NAD⁺) which acts as a cofactor for deacetylation reactivity. The liberated nicotinamide from NAD⁺, generates a novel metabolite o-acetyl-ADP-ribose . SIRT1 can mediate the actions at translational level. Various mechanisms have been proposed to be involved in dysregulation of SIRT1 in cancer cells [Yao, C., et al.2016]. In human breast, lung and prostate cancers SIRT1 is significantly elevated . It plays a role in tumorigenesis by anti-apoptotic activity through oncogene and epigenetic regulator action.[Saunders, L. and E. Verdin 2007]. SIRT1 deacetylates pro-apoptotic proteins such as p53 and promotes cell survival under genotoxic and oxidative stresses [Kojima, K., et al 2010]. It's critical role in multiple aspects of resistance to anti-cancer drugs is also well documented [Duan, K., et al 2015]. Therefore, SIRT1 overexpression is associated with the subsequent higher level of tumor cell proliferation, invasion, and migration [Wang, X., et al 2016].

SIRT1 expression is increased in human colon cancer, acute myeloid leukemia, and some skin can- cers (Bradbury, C. A et al 2005, Hida, Y. et al 2007, Huffman, D. M. et al 2007 and Stunkel, W.2007). SIRT1 , by interacting with and inhibiting p53 may act as tumor promoter (van Leeuwen, I., and Lain, S. 2009). Repression of tumor suppression protein expression and DNA repair protein ,are other roles of SIRT1 in cancer cells. In colon cancer , SIRT1 limits β-catenin signaling while in breast cancer it interacts with BRCA1 signaling . However it has been observed that SIRT1 expression is decreased in ovarian cancer, glioblastoma, and bladder carcinoma (Deng, C. X. 2009). In these cancers , SIRT1 might serve as a tumor suppressor by blocking oncogenic pathways. Thus SIRT1 can serve as a tumor promoter or tumor suppressor, depending on the oncogenic pathways specific to particular tumors.

In hepatocellular carcinoma , SIRT1 was overexpressed in HCC cells and tissues, and significantly promoted the migration and invasion ability of HCC cells by inducing the epithelial and mesenchymal transition[Hao C et al 2014]. This in vivo study also supported the oncogenic functions of SIRT1 in enhancing metastasis[Hao C et al 2014]. Bae et al [Bae HJ et al 2014] found that knockdown of SIRT1 inhibited cell growth by transcriptional deregulation of cell cycle proteins, leading to hypophosphorylation of pRb, which inactivated E2F/ DP1 target gene transcription, and thereby caused the G1/S cell cycle arrest. In addition, miR29c was identified as a suppressor of SIRT1 by comprehensive miRNA profiling and ectopic miR29c expression recapitulated SIRT1 knockdown effects in HCC cells [Bae HJ et al 2014]. To contradict the above findings, Zhang et al [Zhang ZY et al 2015] reported that SIRT1 has anticarcinogenic effects in HCC via the AMPK mammalian target of rapamycin (mTOR) pathway. They evaluated the relationship between p53 mutations and activation of SIRT1 in 252 patients with hepatitis B virus positive HCC and found that activated SIRT1 was associated with a longer recurrence free survival in HCC tissues harbouring mutant p53. He reported that inhibition of SIRT1 increased cell growth, bearing mutated p53, by suppressing AMPK activity and enhancing mTOR activity.The conflicting results from different published data indicated that SIRT1 is multifunctional gene and its biological features are left unsolved.

These above evidence indicates the involvement of SIRT's in regulating three important tumor processes: epithelial-to-mesenchymal transition (EMT), invasion, and metastasis. Many SIRT's are responsible for cellular metabolic reprogramming and drug resistance by inactivating cell death pathways and promoting uncontrolled proliferation. These observations are for the future development of novel tailored SIRT-based cancer therapies.

Wang et al showed that SIRT1 expression was increased in several cancer cell lines, and is generally associated with poor prognosis and overall survival (Wang, C., et al 2017). Vaziri et al reported that SIRT1 interacted with P53, triggering its deacetylation in Lys382 residue, and determined a block of all P53-dependent pathways, leading to uncontrolled cell cycle and inactivation of the apoptotic process (Vaziri, H., et al 2011).

SIRT1 has a function in metastasis and invasiveness in several cancers that has been reported in several studies. Among them ,the deacetylation of many proteins involved in tumor suppressor processes or DNA damage repair, and the inactivation of specific pathways support the role of SIRT1 as a tumor promoter. The role of SIRT1 in the initiation, promotion, and progression of several malignant tumors including prostate cancer (Jung-Hynes, B. et al 2009), breast cancer (Jin, X., et al 2018), lung cancer (Han, L. et al 2013) and gastric cancer (Han, L. et al 2013) are well documented. Wilking el al showed in his in vitro experiments that the inhibition of SIRT1 by treatment with small molecule SIRT1 inhibitors determines a significant decrease in cell growth, proliferation and viability (Wilking, M. J., et al 2014).

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human tissues	qRT-PCR,Western blotting,Luciferase reporter assay H2,H4,H7,H8,H9 Micro-array (Shen ZL et al 2016)	yes	Strong	Yes	Yes	Yes
Human cell lines	Micro-array, qRT- PCR,Western blotting,Luciferase reporter assay (Guo S et al 2020, Bae HJ et al 2014, Zhou J et al 2017, Fu H et al 2018, Lian B et al 2018 Guan Y et al 2017 Yang X et al 2014)	yes	Strong	Yes	Yes	Yes
Mouse	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture	yes	Moderate	Yes	Yes	Yes

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Event: 1172: Increase activation, Nuclear factor kappa B (NF-kB)

Short Name: Increase activation, Nuclear factor kappa B (NF-kB)

Key Event Component

Process	Object	Action
regulation of I-kappaB kinase/NF-kappaB signaling		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:382 - Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Stressors

Name
Reactive oxygen species

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Organ term

Organ term
tissue

Domain of Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage	Evidence
Not Otherwise Specified	Moderate

Sex Applicability

Sex	Evidence
Mixed	

The ROS directly influences NF-kB signalling, resulting in differential production of cytokines and chemokines (McKay and Cidlowski, 1999; Pernis, 2007). NF-kB regulates pro-inflammatory responses that are transcriptionally mediated by NF-kB.

Key Event Description

The NF-kB pathway consists of a series of events where the transcription factors of the NF-kB family play a key role. The proinflammatory cytokine (IL-1beta) can be activated by NF-kB , including Reactive Oxygen Species produced by NADPH oxidase (NOX). Upon pathway activation, the IKK complex will be phosphorylated, which in turn phosphorylates Ikbα. There, this transcription factor can express pro-inflammatory and pro-fibrotic genes. This can be achieved by ROS, IKK enhancer or nuclear translocation enhancer.

How it is Measured or Detected

NF-kB transcriptional activity: Beta lactamase reporter gene assay (Miller et al. 2010). NF-kB transcription: Lentiviral NF-kB GFP reporter with flow cytometry (Moujalled et al. 2012)

NF-κB translocation: RelA-GFP reporter assay (Frederiksson 2012) (Huppelschoten 2017)

IκBa phosphorylation: Western blotting (Miller et al. 2010)

NF-κB p65 (Total/Phospho) ELISA

ELISA for IL-6, IL-8, and Cox

[Event: 112: Antagonism, Estrogen receptor](#)

Short Name: Antagonism, Estrogen receptor

Key Event Component

Process	Object	Action
estrogen receptor activity	estrogen receptor	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	MolecularInitiatingEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

hepatocyte

Domain of Applicability

Taxonomic applicability: Steroid receptors, including ER are thought to have evolved in the chordate lineage (Baker 1997, 2003; Thornton 2001). An ER ortholog has been isolated from a mollusk species, but no ER orthologs have been detected in arthropods or nematodes (Thornton et al. 2003). Broadly speaking, most vertebrates can be expected to have functional ERs, while most invertebrates do not, although there may be exceptions within the mollusk lineage and evolutionarily-related organisms.

Key Event Description

Site of action: The site of action for the molecular initiating event is the liver (hepatocytes).

Responses at the macromolecular level: Estrogen receptor antagonists have been shown to interact with the ligand binding domain of ERs. However, those interactions occur at different contact sites than those of estrogen agonists, leading to a different conformation in the transactivation domain (Brzozowski et al. 1997; Katzenellenbogen 1996).

Characterization of chemical properties: Two broad categories of ER antagonists have been described. Type I, like tamoxifen act as mixed agonists and antagonists. Type II, like ICI164384 are pure antagonists (Katzenellenbogen 1996). Due to their potential utility for treating estrogen-dependent breast cancers and other estrogen-related disease states as well as concerns regarding endocrine disruption, there is an extensive body of literature on the identification and design of chemical structures that act as ER antagonists (e.g., (Brooks et al. 1987; Brooks and Skafar 2004; Lloyd et al. 2006; Sodero et al. 2012; Vedani et al. 2012; Wang et al. 2006).

How it is Measured or Detected

- The BG1luc estrogen receptor transactivation test method for identifying estrogen receptor agonists and antagonists (OECD Test Guideline 457) has been validated by the National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods (NICEATM) and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as an appropriate assay for detecting ER antagonism. (OECD, 2012b).
- Other human ER-based transactivation assays that have been used to detect ERα antagonism include the T47D-Kbluc assay (Wilson et al. 2004); ERα CALUX assay (van der Burg et al. 2010); MELN assay (Witters et al. 2010); and the yeast estrogen screen (YES; (De Boever et al. 2001)). Each of these assays have undergone some level of validation.
- In aquatic ecotoxicology, vitellogenin synthesis in primary fish liver cells and liver slices has also been used to screen for anti-estrogenic activity (e.g., (Bickley et al. 2009; Navas and Segner 2006; Schmieder et al. 2000; Schmieder et al. 2004; Sun et al. 2010)). Although these approaches have generally not been subject to as much formal validation as human ER-based transactivation assays, in the case of fish-specific AOPs linked to this key event, these measures of anti-estrogenicity may be more directly relevant to predicting other key events in the pathway.

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Event: 1457: Induction, Epithelial Mesenchymal Transition

Short Name: EMT

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:241 - Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis	KeyEvent
Aop:206 - Peroxisome proliferator-activated receptors γ inactivation leading to lung fibrosis	KeyEvent
Aop:280 - α-diketone-induced bronchiolitis obliterans	KeyEvent
Aop:347 - Toll-like receptor 4 activation and peroxisome proliferator-activated receptor gamma inactivation leading to pulmonary fibrosis	KeyEvent
Aop:414 - Aryl hydrocarbon receptor activation leading to lung fibrosis through TGF-β dependent fibrosis toxicity pathway	KeyEvent
Aop:415 - Aryl hydrocarbon receptor activation leading to lung fibrosis through IL-6 toxicity pathway	KeyEvent
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Key Event Description

Inflammatory reactions result in the release of various cytokines which in turn can stimulate the transition of epithelial cells to a mesenchymal phenotype acquiring function characteristics of fibroblasts and myofibroblasts.

How it is Measured or Detected

Loss of [E-cadherin](#) and cell polarity is considered to be a fundamental event in epithelial-mesenchymal transition. The simultaneous expression of epithelial (e.g. E-cadherin) and mesenchymal markers (e.g. N-cadherin and vimentin) within the airway epithelium are indicative for ongoing transition (Borthwick et al. 2009, 2010).

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List of Adverse Outcomes in this AOP

Event: 1982: metastatic breast cancer

Short Name: Metastasis, Breast Cancer

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:443 - DNA damage and mutations leading to Metastatic Breast Cancer	AdverseOutcome

Stressors

Name
Ethyl alcohol

Biological Context

Level of Biological Organization

Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage Evidence

Adult High

Sex Applicability

Sex Evidence

Mixed High

Increased metastasis of cancerous cells is known to be highly conserved throughout evolution and is present from humans to invertebrates.

Key Event Description

Biological state:

Dissemination of the cancer cells from one organ to another which is not directly connected to the primary site is called metastasis. It has a crucial role in the prognosis of cancer patients. In the initial stage of metastasis, cancer cells detach from the primary tumor and disseminate in the tissue. Subsequently cancer cells enter the vascular or lymphatic channels (23-25). The establishment of micro-metastasis mainly depends on the survival of the circulating tumor cells (CTCs) inside the lymphatic or blood channels. Extravasation of cancer cells through the vessel wall takes place resulting in the proliferation of cancer cells in the secondary site. Various signalling pathways are involved

in each of the above mentioned process. Few theories have been proposed to explain the mechanism of metastasis. The "organ selection concept" theory suggests that the growth factors establish a successful metastasis in the metastatic site (26,27) whereas the "adhesion theory" proposes the tissue specific adhesion molecules are expressed on endothelial cells of recipient organs which will anchor the migrating cancer cells, providing the a pre-metastatic niche. The role of chemokine receptor has been explained in "chemo-attraction theory" while Paget reported the theory of "seed" for metastatic tumor cells and of "soil" for the secondary site. As per this concept the organ distribution is determined by the site and histopathological type of the primary tumor. The recent understanding suggested, pre-metastatic niche has been indicated to explain metastasis. It is interesting to note that prior to co-localization, the primary tumor induces the micro environment of secondary site by CTCs.

Subsequently, a metastatic niche is generated to support disseminated tumor cells (DTCs) and localize them to develop a metastasis. The most recent theory describes a bidirectional relationship between the primary and secondary sites. According to this theory, the surviving cancer cells in the metastatic tumor can return to the primary site to promote the primary tumor progression (28,29). Efficient and direct blood flow can explain the probability of metastasis to the specific organs like hepatic metastasis in patients with colon cancer which receive direct blood flow from the primary site. Vascular permeability is also the other factor which significantly promotes extravasation at the metastatic site. However at present, understanding of molecular mechanisms of metastasis remains incomplete.

Biological compartment

Organs, Cellular

Role in general biology

Epithelial- mesenchymal transition (EMT) and its reverse mesenchymal-epithelial transition (MET) are characteristics of cellular plasticity during embryogenesis and tumor metastasis (30). There has been decreased expression of E-cadherin and β -catenin and elevated expression levels of vimentin, fibronectin and N-cadherin in EMT (31). In cancers, EMT is a major process by which cancer cells lose their epithelial characteristics to acquire mesenchymal-like properties. Tumor cell migration is a pre-requisite for the metastatic process in which, EMT is the most critical step to initiate metastasis including metastasis to lymph nodes (32). During EMT, cancer cells lose their cell-to-cell junctions and cellular polarity via multiple signaling pathways which increase the motilities and invasive phenotype of them (33). Cleavage of E-cadherin mediated by the MMPs increases the tumor cell motility and invasion. Apart from this, EMT has a key role in drug resistance. This is supported by the finding that high levels of vimentin was found in adriamycin and vinblastine resistant breast cancer cell lines (34). EMT promotes CSCs motility, cancer cell invasion, tumor metastasis and recurrence and drug resistance. Expression of stem cell like markers and formation of tumor spheres by CSCs are enhanced by EMT process. CSCs acquire mesenchymal features by undergoing EMT phenomenon. By acquiring mesenchymal features, CSCs become resistant to anti-cancer therapies; hence, they can survive and cause cancer recurrence. In addition to this, CSCs invade to the adjacent stromal tissues, enter the vascular channels, and finally reach the distant organs. In the target organs, CSCs cause MET phenomenon which results in the acquisition of epithelial characteristics. MET phenomenon also increases the cell-to-cell attachment, cancer cells proliferation and differentiation to form metastatic lesions (35). Altogether, EMT induces CSC properties and metastatic activities. On the other hand, EMT and CSCs collaborate in invasion capacity hence targeting the EMT/CSC phenotype can be a therapeutic approach for the treatment of metastasis and tumor recurrence (36).

EMT programs are regulated by a network of signal- ling pathways that involve components such as growth factors (transforming growth factor- β [TGF- β], epider- mal growth factor [EGF]) and their associated signalling proteins (Wnt, Notch, Hedgehog, nuclear-factor kappa B [NF- κ B], extracellular signal-regulated kinase [ERK], and phosphatidylinositol 3-kinase [PI3K]/Akt) in response to stresses involved in tumorigenesis, including hypoxia, oncogenic or metabolic stress, inflammation, and physical constraints [37-41].

These signals activate EMT-inducing transcription factors, including Snail/Slug, ZEB1/5EF1, ZEB2/SIP1, Twist1/ 2, and E12/E47 [42-44]. EMT-inducing transcription factors regulate the expression of proteins involved in cell polarity, cell-cell contact, cytoskeletal structural maintenance, and extracellular matrix (ECM) degradation, and they sup- press key epithelial genes. Loss of E-cadherin is considered a hallmark of EMT; these EMT-inducing transcription factors bind to E-box elements in the E-cadherin gene promoter to repress its transcription. Of particular note, Snail is an early marker of EMT that is involved in the initial cell-migratory phenotype, and it occasionally induces other factors .

During EMT, epithelial cells reorganize cytoskeleton and resolve cell-cell junctions, which are accompanied with switching off the expression of epithelial markers and turning on mesenchymal genes. Although changes in epithelial and mesenchymal markers during EMT can vary significantly in different biologic contexts, a network of transcription factors, including TWIST1/2, SNAIL1/2, ZEB1/2, and FOXC2, are consistently required to orchestrate the EMT program (45). The expression of these transcription factors is associated with poor prognosis and distant metastasis in various human cancers has been documented in various studies. (46). Besides its role in promoting tumor cell invasion, EMT is shown to confer tumor cells with resistance to apoptosis and anoikis (47), thus allowing cell survival in the blood stream after intravasation. EMT could also facilitate tumor cells' escape from the senescence program, especially through TWIST1 and ZEB1 (48,49). Furthermore, EMT has been shown to cancer cells with cancer stem cell (CSC)-like features, which further aid tumor dormancy and chemo resistance (50,51). Tumor samples or experimental tumor xenograft models have provided convincing evidence for the activation of EMT in various primary epithelial tumors in various studies. Interestingly, more recent studies reveal a dynamic requirement of EMT in tumor metastasis: activation of EMT promotes local tumor invasion, intravasation, and extravasation of the systemic circulation, whereas reversion of EMT is essential to establish macrometastasis in distant organs (52,53).

How it is Measured or Detected

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Cell line, humans, Human cell line studies	qRT-PCR, Luciferase reporter assay, immunoblotting, immunoprecipitation, cell invasion assay, cell migration assay, bioluminescence imaging, wound healing assay, Wound scratch & Transwell assay, Microarray, Immunofluorescence, Immunohistochemistry,	+	Strong	Yes	Yes	Yes

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2608: Increased, DNA damage and mutation leads to Inadequate DNA repair

AOPs Referencing Relationship				
AOP Name		Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer		adjacent	High	High
Evidence Supporting Applicability of this Relationship				
Taxonomic Applicability				
Term	Scientific Term	Evidence	Links	
rat	Rattus norvegicus	High	NCBI	
mouse	Mus musculus	High	NCBI	
Life Stage Applicability				
Life Stage		Evidence		
Not Otherwise Specified		High		
Sex Applicability				
Sex	Evidence			
Female	High			
In any eukaryotic or prokaryotic cell, oxidative DNA damage can develop and overwhelm the cell's repair processes. This KER has been seen in mammalian cells, yeast, and bacteria, among other places.				
Key Event Relationship Description				
Upstream event: Increased, DNA damage and mutation				
Downstream event: DNA repair mechanism, Reduced				
Evidence Supporting this KER				
DNA damage leading to inadequate repair mechanisms :				
- As a result of DNA damage, DNA repair activities change. A variety of genotoxic agents, such as N-nitrosodimethylamine, aflatoxin B1, and 2-acetylaminofluorene induce the protein, O6-Alkylguanine-DNA alkyltransferase (ATase), which is responsible for repair of DNA alkylation damage in rats (O'Connor, 1989; Chinnasamy et al.,1996). Grombacher and Kaina (1996) reported an increased human ATase mRNA expression by alkylating agents like N-methyl-N-nitro-N-nitrosoguanidine and methyl methanesulphonate and by ionizing radiation via the induction of the ATase promoter. ATase mRNA expression was increased in response to treatment with 2-acetylaminofluorene in rat liver (Potter et al., 1991; Chinnasamy et al., 1996). In another study, it was demonstrated that ATase gene induction is p53 gene-dependent: ATase activity was induced in mouse tissues following γ-irradiation in p53 wild type mice, but not in p53 null animals (Rafferty et al., 1996). - Alkylating agents and X-rays also induce DNA glycosylase, alkylpurine-DNA-N-glycosylase (APNG) (Lefebvre et al., 1993; Mitra and Kaina, 1993). - As a consequence of these and other observations, there is considerable interest in investigating DNA repair modulation as a possible risk factor in carcinogenesis.				
- Due to low levels of reactive oxygen species (ROS) and other free radicals generated by endogenous redox reactions, oxidative DNA lesions are present in the cell at steady state. - The most important oxidative DNA lesions include 7, 8-dihydro-8-oxo-deoxyGuanine (8-oxo-dG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPydG), and thymidine glycol (Tg). - Under homeostatic settings, cells can control the amount of free radicals in the environment and quickly repair oxidised DNA bases with basal repair mechanisms, preventing irreparable damage (Swenberg et al., 2011). Oxidative DNA lesions are mainly repaired by base excision repair (BER) initiated by DNA glycosylases such as oxoguanine glycosylase 1 (OGG1), endonuclease III homologue 1 (NTH1), and Nei-like DNA glycosylases (NEIL 1/2), which detect and remove damaged bases. - Endonucleases or lyases cleave abasic sites, resulting in transitory single-strand breaks (SSB) that can be repaired in either short-patch or long-patch fashion. To a lesser extent, nucleotide excision repair (NER) is involved in repairing oxidised bases. (Shafirovich et al., 2016). - Increased levels of free radicals or exposure to oxidising agents can increase the number of oxidative DNA lesions and overload repair mechanisms, lowering repair quality. If the repair mechanisms are weakened, oxidative lesions might build up (insufficient repair), resulting in erroneous base pairing during replication or incomplete repair (indicated by accumulation of repair intermediates) (Markkanen et al., 2017).				
Biological Plausibility				
BER and, to a lesser extent, NER are used to repair oxidative DNA damage. Previous research has found thresholded dose-response curves in oxidative DNA damage and attributed these findings to a lack of repair capability at the curve's inflection point (Gagne et al., 2012; Seager et al., 2012). Following chemical exposures, in vivo, a rise and buildup of oxidative DNA lesions was seen despite the activation of BER, suggesting poor repair of oxidative DNA lesions beyond a certain level(Ma et al., 2008).				
OGG1 and NTH1, the glycosylases that initiate the BER of 8-oxo-dG and thymine glycol (Tg) lesions, respectively, are bifunctional, containing both glycosylase and lyase activities. By cleaving the glycosidic link, the glycosylase eliminates the oxidised guanine and creates an apurinic site.The lyase then cleaves the phosphodiester bond 5' to the AP site; a transient SSB is created for further processing in BER (Delaney et al., 2012). Abasic sites created by OGG1 and other glycosylases are also processed by apuric/apyrimidinic endonucleases (APE1) to create the 5' nick (Allgayer et al., 2016).				
Previous research has shown that an imbalance in any of the BER's several phases might result in an accumulation of repair intermediates and failed repair. Given that OGG1 is slower than other glycosylases in releasing its catalytic product, a disproportionate rise in oxidative DNA lesions compared to the quantity of accessible OGG1 is highly likely to result in an imbalance between lesions and the BER initiating step (Brenerman et al., 2014). As a result, oxidative lesions would begin to accumulate. Furthermore, overexpression of OGG1 and NTH1 has been linked to the accumulation of SSB, suggesting that the unbalanced lyase activity causes an excess of SSB intermediates(Yang et al., 2004; Yoshikawa et al., 2015; Wang et al., 2018).				
Increases in oxidative lesions may result in more lesions and repair intermediates being produced in close proximity. Previous research on mammalian cell extracts has shown that when oxidative damages occur in parallel or opposite each other, repair effectiveness is reduced.OGG1 showed reduced binding to 8-oxo-dG near an AP site incision. Furthermore, the OGG1-8-oxo-dG complex has been observed to hinder the repair of neighbouring AP site incision, delaying the completion of BER; It's been claimed that this interaction between BER enzymes causes a buildup of oxidative lesions and repair intermediates(Pearson et al., 2004; Budworth et al., 2005; Bellon et al., 2009; Yoshikawa et al., 2015; Sharma et al., 2016).				
If oxidative lesions persist in the genome due to insufficient repair, incorrect base insertion opposite unrepaired oxidative DNA lesions may occur during replication. This is a well-established event. For example, 8-oxo-dG and FaPydG, the two most prominent oxidative DNA lesions, are able to form base pairs with dATP, giving rise to G:C→T:A transversions after subsequent DNA synthesis (Freudenthal et al., 2013; Gehrke et al., 2013; Markkanen, 2017). Replicative DNA polymerases such as DNA polymerase α, δ, and ε (pol α, δ, ε) have a poor ability to extend the DNA strand past 8-oxo-dG:dCTP base pairs and may cause replication to stall or incorrectly insert dATP opposite 8-oxo-dG (Hashimoto et al., 2004; Markkanen et al., 2012). In stalled replication forks, repair polymerases may be recruited to perform translesion DNA synthesis (TLS). Human Y-family DNA polymerases (Rev 1, pol κ, ι, and η) are DNA repair polymerases mainly involved in TLS in stalled replication forks. However, TLS is not free of error and its accuracy differs for each repair polymerase. For example, it is known that pol κ and η perform TLS across 8-oxo-dG and preferentially insert dATP opposite the lesion, generating G:C→T:A transversions. The error-prone nature of bypassing unrepaired oxidative lesions has been described in many previous studies and reviews (Greenberg, 2012; Maddukuri et al., 2014; Taggart et al., 2014; Shah et al., 2018).				
Repair by OGG1 requires 8-oxo-dG:dC base pairing, thus, it is unable to repair 8-oxo-dG:dA mispairing in newly synthesized strands. The repair of 8-oxo-dG:dA base pairs post-replication is performed by MUT Y homologue, MYH, an adenine DNA glycosylase. However, the removal of dA instead of the damaged guanine may lead to futile cycles of BER because: 1) another dA is often inserted opposite the lesion, or 2) BER ligases have a poor ability of ligating the 3'end of dC opposite 8-oxo-dG (Hashimoto et al., 2004; Caglayan and Wilson, 2015). Accumulated 8-oxo-dG may be more resistant to repair post-replication due to this futile BER.				
Empirical Evidence				
<u>In vitro studies demonstrating dose and temporal concordance, or essentiality</u>				
- Human normal hepatocytes (HL-7702) were subjected to escalating doses of N,N-dimethylformamide for 24 hours (C. Wang et al., 2016) - At all concentrations, a concentration-dependent increase in ROS was detected; the rise was statistically significant when compared to control (6.4, 16, 40, 100 mM) - Until the highest two concentrations (40 and 100 mM), no significant rise in 8-oxodG was seen, indicating inadequate repair at these dosages. - Excision repair genes (XRCC2 and XRCC3) were considerably up-regulated at 6.4 and 16 mM, well below the doses that significantly produced 8-oxodG, indicating that adequate DNA repair was possible at these low concentrations. - These findings show that repair is competent at low concentrations (removing 8-oxodG quickly), but that repair is swamped (i.e., insufficient) at larger doses, where 8-oxodG greatly increases. - AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to varying doses of ultraviolet A (UVA) radiation (Dahle et al., 2008) - Formamidopyrimidine glycosylase (Fpg)-sensitive sites were quantified using alkaline elution after increasing repair times (0, 1, 2, 3, 4 h) following 100 kJ/m² UVA irradiation - OGG1-overexpressing AS52 cells (OGG1+): Fpg-sensitive sites reduced to 71% within half an hour and down to background levels at 4h - Wild type AS52 cells: at 4h, 70% of the Fpg-sensitive sites remained, indicating accumulation of oxidative lesions				

- Mutations in the *Gpt* gene was quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m² UVA irradiation
 - G:C→T:A mutations in UVA-irradiated OGG1+ cells were completely eliminated (thus, repair was sufficient when repair overexpressed).
 - G:C→T:A mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-oxo-dG.
 - The importance of 8-oxo-dG production in oxidative DNA damage-induced G to T transversion mutations is further demonstrated by the above result.
- HL-60 human leukemia cells were irradiated with X-rays at a rate of 0.5 Gy/min for increasing durations (i.e., increasing doses). 8-OHdG levels were quantified by HPLC as number of 8-OHdG per 10⁶ deoxyguanosine (Li et al., 2013)
 - No increase in 8-OHdG was observed up to 2 Gy (sufficient repair at low doses), above which the level of lesions increased linearly up to 20 Gy (insufficient repair)
 - In the same study described below, a thresholded dose-response curve, indicating of overwhelmed repair processes, was also seen in mouse liver.

In vivo studies demonstrating dose concordance

- Two groups of 5-week-old C57BL/6J mice were exposed to increasing doses of X-rays at a rate of 0.5 Gy/min (200 kV, 12 mA). The livers of one group of mice were obtained immediately after exposure, while urine samples were collected over the course of 24 hours after irradiation in the second group of mice (Li et al., 2013).
 - The amount of 8-OHdG in mouse liver DNA was measured by HPLC and expressed as 8-OHdG per 10⁶ deoxyguanosine
 - Between 0 and 0.5 Gy, no increase in lesions was observed
 - Between 0.5 and 30 Gy, a linear dose-response in 8-OHdG was observed
 - The thresholded dose-response curve was concordant in the urine samples; no increase in urinary 8-OHdG (8-OHdG/creatinine (ng/mg)) was observed between 0 and 0.1 Gy but between 0.1 and 5 Gy, the number of lesions increased linearly with dose
- For 30 days, male Sprague-Dawley rats were fed 0.5 mmol aniline/kg/day. Spleen tissues were used to acquire genomic DNA, nuclear extracts, and mitochondrial extracts (Ma et al., 2008).
 - 8-OHdG was measured on digested genomic DNA using an enzyme-linked immunosorbent assay (ELISA). The number of lesions in aniline-fed rats was 2.8 times higher than in control rats.
 - OGG1 activity was measured in both nuclear and mitochondrial extracts of aniline-treated rats, with a 1.32-fold and 1.15-fold increase in enzyme activity (both significant; p<0.05) seen in the corresponding extracts.
 - Western blotting was used to assess the OGG1 enzyme content in the extracts; the increase in OGG1 content in aniline-treated rats was consistent with the OGG1 activity assay.
 - The amount of 8-OHdG rose despite an increase in OGG1 enzyme concentration and activity.
- These findings show that because 8-oxodG adducts are rapidly eliminated, repair is sufficient at low doses. 8-oxo-dG begins to significantly increase at larger concentrations, indicating that repair is being overwhelmed (i.e., insufficient).

Uncertainties and Inconsistencies

Despite the fact that OGG1's dual activity as a glycosylase and lyase has been widely acknowledged and proved experimentally, investigations suggest that apurinic endonuclease 1 is primarily responsible for the cleavage of phosphodiester link 5' to the lesion (APE1) (Allgayer et al., 2016; R. Wang et al., 2018). In rare circumstances, APE1 may be the primary driver of BER intermediate buildup. According to some research, OGG1 is involved in the repair of non-transcribed strands but isn't essential for transcription-coupled 8-oxo-dG repair.; Le Page et al. reported efficient repair of 8-oxo-dG in the transcribed sequence in *Ogg1* knockout mouse cells (Le Page et al., 2000). Furthermore, the repair of 8-oxo-dG is influenced by the sequences surrounding it; the location of the lesions may have a negative impact on repair effectiveness. (Pastoriza-Gallego et al., 2007). We note that the study by Allgayer et al. was investigating the fate and effect of 8-oxo-dG during transcription; repair mechanism may vary by situation and availability of repair enzymes at the time.

Quantitative Understanding of the Linkage

The specific relationship between oxidative DNA lesions and when repair is regarded insufficient has yet to be determined; this relationship will most likely differ between cell types and tissues, making it difficult to define. There are computational models of 8-oxo-dG repair kinetics.

To explore the rate of clearance of BER repair intermediates, Sokhansanj and Wilson III [2004] used a quantitative model of BER and a literature estimate for the rate of generation of endogenous 8-oxo-dG (Sokhansanj and Wilson III, 2004).

- The OGG1, AP lyases, polymerases, and ligases activities were incorporated in the BER model, which used Michaelis-Menten enzyme kinetics.
- The model assumed that endogenous oxidative lesions formed at a rate of 500 8-oxo-dG/day.
 - Based on the information above, it was calculated that after a sudden increase in 8-oxo-dG to 20,000 8-oxo-dG/cell, the total amount of repair intermediates would revert to baseline in 4000 seconds (less than 1 hour)
 - This model also assumed that OGG1 was available in excess
- The glycosylase reaction kinetics of OGG1 (a bifunctional glycosylase/lyase) were reported to increase when APE1 (AP site endonuclease) was present. It's thought to be because the two enzymes work together.
- When OGG1 kinetics were reduced by tenfold, 8-oxo-dG increased tenfold, while no other repair intermediates increased.
- Quantitative understanding is represented as below;

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Rat	Quantification of ATase activity – BSA method APNG assay, OXOG glycosylase activity assay, Western immunoblotting, Immunohistochemical detection of ATase (Kotova et al.,2013)	Yes	Strong	Yes	Yes	Yes
Rat cell line	Flow cytometric micronucleus assay, Cell cycle analysis, Replication fork elongation assay, Cytotoxicity assay, Recombination assay, (Panida et al.,2001)	Yes	Strong	Yes	Yes	Yes
mouse	FISH karyotyping, Invivo point mutation assay, Whole genome sequencing of HSC clones (Garayacoechea et al.,2012)	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Linear increase in DNA damage was noted following exposure to the stressor.

Time-scale

Changes were noted within 24 hours of treatment with the stressor, however after withdrawal of the stressor, persisted for 3-4 weeks.

The acute ethanol dose significantly inhibited O⁶-alkylguanine-DNA alkyltransferase (ATase) activity by 21–32% throughout the 24-h post-treatment period and this was confirmed by immunohistochemical detection of the ATase protein in hepatic nuclei. Twelve hours after the ethanol treatment, the activities of the DNA glycosylases, alkylpurine-DNA-*N*-glycosylase (APNG) and 8-oxoguanine-DNA glycosylase (OXOG glycosylase) were each increased by ~44%. In contrast, when given chronically via the liquid diet, ethanol initially had no effect on ATase activity, but after 4 weeks ATase activity was increased by 40%. Following ethanol withdrawal, ATase activity remained elevated for at least 12 h, but, by 24 h, the activity had fallen to the uninduced control level. DNA glycosylase activities were again affected differently. After 1 week of dietary ethanol exposure, there was no effect on APNG activity but it was inhibited by 19% at 4 weeks. OXOG glycosylase activity, on the other hand, was increased by 53% after 1 week, but decreased by 40% after 4 weeks.

Known modulating factors

DNA repair mechanism depends on the cell type,age of the cell and extra cellular environment.

Known Feedforward/Feedback loops influencing this KER

Not found to the best of our knowledge.

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Relationship: 164: Inadequate DNA repair leads to Increase, Mutations

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Alkylation of DNA in male pre-meiotic germ cells leading to heritable mutations	adjacent	High	Moderate
Alkylation of DNA leading to cancer 2	adjacent	High	Moderate
Alkylation of DNA leading to cancer 1	non-adjacent	High	Moderate
Oxidative DNA damage leading to chromosomal aberrations and mutations	adjacent	High	Low
Deposition of energy leading to lung cancer	adjacent	Moderate	Moderate
Bulky DNA adducts leading to mutations	adjacent		
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
Life Stage Applicability			
	Life Stage	Evidence	
	All life stages	High	
Sex Applicability			
	Sex	Evidence	
	Unspecific	High	
The domain of applicability is multicellular eukaryotes (Lieber, 2008; Hartlerode & Scully, 2009), plants (Gorbulnova, 1997; Puchta, 2005), certain strains of bacteria such as <i>Mycobacteria</i>, <i>Pseudomonas</i>, <i>Bacillus</i> and <i>Agrobacterium</i> (Shuman & Glickman, 2007), and yeast (Wilson & Lieber, 1999).			
All organisms, from prokaryotes to eukaryotes, have DNA repair systems. Indeed, much of the empirical evidence on the fundamental principles described in this KER are derived from prokaryotic models. DNA adducts can occur in any cell type, and may or may not be repaired, leading to mutation. While there are differences among DNA repair systems across eukaryotic taxa, all species develop mutations following excessive burdens of DNA lesions like DNA adducts. Theoretically, any sexually reproducing organism (i.e., producing gametes) can also acquire DNA lesions that may or may not be repaired, leading to mutations in gametes.			
Key Event Relationship Description			
Insufficient repair results in the retention of damaged DNA that is then used as a template during DNA replication. During replication of damaged DNA, incorrect nucleotides may be inserted, and upon replication these become 'fixed' in the cell. Further replication propagates the mutation to additional cells. For example, it is well established that replication of alkylated DNA can cause insertion of an incorrect base in the DNA duplex (i.e., mutation). Replication of non-repaired O4 thymine alkylation leads primarily to A:T→G:C transitions. Retained O6 guanine alkylation causes primarily G:C→A:T transitions. For repairing DNA double strand breaks (DSBs), non-homologous end joining (NHEJ) is one of the repair mechanisms used in human somatic cells (Petrini et al., 1997; Mao et al., 2008). However, this mechanism is error-prone and may create mutations during the process of DNA repair (Little, 2000). NHEJ is considered error-prone because it does not use a homologous template to repair the DSB. The NHEJ mechanism involves many proteins that work together to bridge the DSB gap by overlapping single-strand termini that are usually less than 10 nucleotides long (Anderson, 1993; Getts & Stamato, 1994; Rathmell & Chu, 1994). Inherent in this process is the introduction of errors that may result in mutations such as insertions, deletions, inversions, or translocations.			
Evidence Supporting this KER			
Biological Plausibility			
If DNA repair is able to correctly and efficiently repair DNA lesions introduced by a genotoxic stressor, then no increase in mutation frequency will occur. For example, for alkylated DNA, efficient removal by AGT will result in no increases in mutation frequency. However, above a certain dose AGT becomes saturated and is no longer able to efficiently remove the alkyl adducts. Replication of O-alkyl adducts leads to mutation. The evidence demonstrating that replication of unrepaired O-alkylated DNA causes mutations is extensive in somatic cells and has been reviewed (Basu and Essigmann 1990; Shrivastav et al. 2010); specific examples are given below. It is important to note that not all DNA lesions will cause mutations. It is well documented that many are bypassed error-free. For example, N-alkyl adducts can quite readily be bypassed error-free with no increase in mutations (Philippin et al., 2014).			
Inadequate repair of DSB			
Collective data from tumors and tumor cell lines has emerged that suggests that DNA repair mechanisms may be error-prone (reviewed in Sishc et al., 2017) (Sishc & Davis, 2017). NHEJ, the most common pathway used to repair DSBs, has been described as error-prone. The error-prone nature of NHEJ, however, is thought to be dependent on the structure of the DSB ends being repaired, and not necessarily dependent on the NHEJ mechanism itself (Bétermier et al., 2014). Usually when perfectly cohesive ends are formed as a result of a DSB event, ligase 4 (LIG4) will have limited end processing to perform, thereby keeping ligation errors to a minimum (Waters et al., 2014). When the ends are difficult to ligate, however, the resulting repair may not be completed properly; this often leads to point mutations and other chromosomal rearrangements. It has been shown that approximately 25 - 50% of DSBs are misrejoined after exposure to ionizing radiation (Löbrich et al., 1998; Kuhne et al., 2000; Löbrich et al., 2000). Defective repair mechanisms can increase sensitivity to agents that induce DSBs and lead eventually to genomic instability (reviewed in Sishc et al., (2017)).			
Activation of mutagenic DNA repair pathways to withstand cellular or replication stress either from endogenous or exogenous sources can promote cellular viability, albeit at a cost of increased genome instability and mutagenesis (Fitzgerald et al., 2017). These salvage DNA repair pathways including, Break-induced Replication (BIR) and Microhomology-mediated Break-induced Replication (MMBIR). BIR repairs one-ended DSBs and has been extensively studied in yeast as well as in mammalian systems. BIR and MMBIR are linked with heightened levels of mutagenesis, chromosomal rearrangements and ensuing genome instability (Deem et al., 2011; Sakofsky et al., 2015; Saini et al., 2017; Kramara et al., 2018). In mammalian genomes BIR-like synthesis has been proposed to be involved in late stage Mitotic DNA Synthesis (MiDAS) that predominantly occurs at so-called Common Fragile Sites (CFSs) and maintains telomere length under s conditions of replication stress that serve to promote cell viability (Minocherhomji et al., 2015; Bhowmick et al., 2016; Dilley et al., 2016).			
Empirical Evidence			
INSUFFICIENT REPAIR OF ALKYLATED DNA			
Evidence in somatic cells Empirical evidence to support this KER is primarily from studies in which synthetic oligonucleotides containing well-characterized DNA lesions were genetically engineered in viral or plasmid genomes and subsequently introduced into bacterial or mammalian cells. Mutagenicity of each lesion is ascertained by sequencing, confirming that replication of alkylated DNA (i.e., unrepaired DNA) causes mutations in addition to revealing the important DNA repair pathways and polymerases involved in the process. For example, plasmids containing O6-methyl or O6-ethylguanine were introduced into AGT deficient or normal Chinese hamster ovary cells (Eilison et al. 1989). Following replication, an increase in mutant fraction to 19% for O6-methylguanine and 11% for O6-ethylguanine adducts was observed in AGT deficient cells versus undetectable levels for control plasmids. The relationship between input of alkylated DNA versus recovered mutant fractions revealed that a large proportion of alkyl adducts were converted to mutations in the AGT deficient cells (relationship slightly sublinear, with more adducts than mutations). The primary mutation occurring was G:C-A:T transitions. The results indicate that replication of the adducted DNA caused mutations and that this was more prevalent with reduced repair capacity. The number of mutations measured is less than the unrepaired alkyl adducts transfected into cells, supporting that insufficient repair occurs prior to mutation. Moreover, the alkyl adducts occur prior to mutation formation, demonstrating temporal concordance. Various studies in cultured cells and microorganisms have shown that the expression of AGT/MGMT (repair machinery – i.e., decrease in KE1) greatly reduces the incidence of mutations caused by exposure to methylating agents such as MNU and MNNG (reviewed in Kaina et al. 2007; Pegg 2011). Thomas et al. (2013) used O6-benzylguanine to specifically inhibit MGMT activity in AHH-1 cells. Inhibition was carried out for one hour prior to exposure to MNU, a potent alkylating agent. Inactivation of MGMT resulted in increased MNU-induced HPRT (hypoxanthine-guanine phosphoribosyltransferase) mutagenesis and shifted the concentrations at which induced mutations occurred to the left on the dose axis (10 fold reduction of the lowest observed genotoxic effect level from 0.01 to 0.001 µg/ml). The ratio of mutants recovered in DNA repair deficient cells was 3-5 fold higher than repair competent cells at concentrations below 0.01 µg/ml, but was approximately equal at higher concentrations, indicating that repair operated effectively to a certain concentration. Only at this concentration (above 0.01 µg/ml when repair machinery is overwhelmed and repair becomes deficient) do the induced mutations in the repair competent cells approach those of repair deficient. Thus, induced mutation frequencies in wild type cells are suppressed until repair is overwhelmed for this alkylating agent. The mutations prevented by MGMT are predominantly G:C-A:T transitions caused by O6-methylguanine. Evidence in germ cells That saturation of repair leads to mutation in spermatogonial cells is supported by work using the OECD TG488 rodent mutation reporter assay in sperm. A sub-linear dose-response was found using the lacZ MutaMouse assay in sperm exposed as spermatogonial stem cells, though the number of doses was limited (van Delft and Baan 1995). This is indirect evidence that repair occurs efficiently at low doses and that saturation of repair causes mutations at high doses. Lack of additional data motivated a dose-response study using the MutaMouse model following both acute and sub-chronic ENU exposure by oral gavage (O'Brien et al. 2015). The results indicate a linear dose-response for single acute exposures, but a sub-linear dose-response occurs for lower dose sub-chronic (28 day) exposures, during which mutation was only observed to occur at the highest dose. This is consistent with the expected pattern for dose-response based on the hypothesized AOP. Thus, this sub-linear curve for mutation at low doses following sub-chronic ENU exposure suggests that DNA repair in spermatogonia is effective in preventing mutations until the process becomes overwhelmed at higher doses. Mutation spectrum: Following exposure to alkylating agents, the most mutagenic adducts to DNA in pre-meiotic male germ cells include O6-ethylguanine, O4-ethylthymine and O2-ethylthymine (Beranek 1990; Shelby and Tindall 1997). Studies on sperm samples collected post-ENU exposure in transgenic rodents have shown that 70% of the observed mutations are at A:T sites (Douglas et al. 1995). The mutations observed at G:C base pairs are almost exclusively G:C-A:T transitions, presumably resulting from O6-ethylguanine. It is proposed that the prevalence of mutations at A:T basepairs is the result of efficient removal of O6-alkylguanine by AGT in spermatogonia, which is consistent with observation in human somatic cells (Bronstein et al. 1991; Bronstein et al. 1992). This results in the majority of O6-ethylguanine adducts being removed, leaving O4- and O2-ethylthymine lesions to mispair during replication. Thus, lack of repair predominantly at thymines and guanines at increasing doses leads to mutations in these nucleotides, consistent with the concordance expected between diminished repair capabilities at these adducts and mutation induction (i.e., concordance relates to seeing these patterns across multiple studies, species and across the data in germ cells and offspring).			
Inadequate repair of oxidative DNA lesions: In vitro studies			
- AS52 Chinese hamster ovary cells (wild type and OGG1-overexpressing) were exposed to kJ/m² UVA radiation (Dahle et al., 2008). - Mutations in the gpt gene were quantified in both wild type and OGG1+ cells by sequencing after 13-15 days following 400 kJ/m² UVA irradiation - G:C-A:T mutations in UVA-irradiated OGG1+ cells were completely eliminated - G:C-A:T mutation frequency in wild type cells increased from 1.8 mutants/million cells to 3.8 mutants/million cells following irradiation – indicating incorrect repair or lack of repair of accumulated 8-			

- oxo-dG
 - Elevated levels of OGG1 was able to prevent G:C-A:T mutations, while the OGG1 levels in wild type cells was insufficient, leading to an increase in mutants (demonstrates inadequate repair leading to mutations)
- Xeroderma pigmentosum complementation group A (XPA) knockout (KO) and wild type TSCER122 human lymphoblastoid cells were transfected with TK gene-containing vectors with no adduct, a single 8-oxo-dG, or two 8-oxo-dG adducts in tandem (Sassa et al., 2015).
 - XPA is a key protein in nucleotide excision repair (NER) that acts as a scaffold in the assembly the repair complex.
 - Mutation frequency was determined by the number of TK-revertant colonies
 - Control vector induced a mutation frequency of 1.3% in both WT and XPA KO
 - Two 8-oxo-dG in tandem on the transcribed strand were most mutagenic in XPA KO, inducing 12% mutant frequency compared to 7% in WT
 - For both XPA KO and WT, G:C-A:T transversion due to 8-oxo-dG was the most predominant point mutation in the mutants
 - The lack of a key factor in NER leading to increased 8-oxo-dG-induced transversions demonstrates insufficient repair leading to increase in mutations

Inadequate repair of oxidative DNA lesions: In vivo studies in mice

- Spontaneous mutation frequencies in the liver of Ogg1-deficient (-/-) Big Blue mice was measured at 10 weeks of age (Klungland et al., 1999).
 - Mutation frequencies were 2- to 3-fold higher in the *Ogg1*^{-/-} mice than in wild type
 - Of the 16 base substitutions detected in *Ogg1*^{-/-} mutant plaques analyzed by sequencing, 10 indicated G:C-A:T transversions consistent with the known spectrum of mutation
 - The results support that insufficient repair of oxidized bases leads to mutation.
- *Ogg1* knockout (*Ogg1*^{-/-}) in C57BL/6J mice resulted in 4.2-fold and 12-fold increases in the amount of 8-oxo-dG in the liver compared to wild type at 9 and 14 weeks of age, respectively (Minowa et al., 2000).
 - In these mice, there was an average of 2.3-fold increase in mutation frequencies in the liver (measured between 16-20 weeks)
 - 57% of the observed base substitutions were G:C-A:T transversions, while 35% in wild type mice corresponded to this transversion.
 - Approximately 70% of the increase in mutation frequency was due to G to T transversions.
 - Concordantly, KBrO3 treatment resulted in a 2.9-fold increase in mutation frequency in the kidney of *Ogg1*^{-/-} mice compared to KBrO3-treated wild type (Arai et al., 2002).
 - G:C-A:T transversions made up 50% of the base substitutions in the *Ogg1*^{-/-} mice.
 - Heterozygous *Ogg1* mutants (*Ogg1*^{+/-}) retained the original repair capacity, where no increase in 8-oxo-dG lesions was observed in the liver at 9 and 14 weeks (Minowa et al., 2000).
 - This observation was consistent even after KBrO3 treatment of the mice (Arai et al., 2002).
 - From these results, we can infer that OGG1 proteins are present in excess and that one functional copy of the gene is sufficient in addressing endogenous and, to a certain degree, chemical-induced oxidative DNA lesions.

Inadequate Repair of DSB

Empirical data obtained for this KER moderately supports the idea that inadequate DNA repair increases the frequency of mutations. The evidence presented below related to the inadequate repair of DSBs is summarized in table 5, [here \(click link\)](#). The review article by Sishc & Davis (2017) provides an overview of NHEJ mechanisms with a focus on the inherently error-prone nature of DSB repair mechanisms, particularly when core proteins of NHEJ are knocked-out. Another review also provides an overview of DSB induction, the repair process and how mutations may result, as well as the biological relevance of misrepaired or non-repaired DNA damage (Sage & Shikazono, 2017).

Dose and Incidence Concordance

There is evidence in the literature suggesting a dose/incidence concordance between inadequate DNA repair and increases in mutation frequencies. Evidence presented below related to the dose-response of mutation frequencies is summarized in table 2, [here \(click link\)](#). In response to increasing doses from a radiation stressor, dose-dependent increases in both measures of inadequate DNA repair and mutation frequency have been found. In an analysis that amalgamated results from several different studies conducted using in vitro cell-lines, the rate of DSB misrepair was revealed to increase in a dose-dependent fashion from 0 - 80 Gy, with the mutation rate also similarly increasing from 0 - 6 Gy (McMahon et al., 2016). Additionally, using a plant model, it was shown that increasing radiation dose from 0 - 10 Gy resulted in increased DNA damage as a consequence of inadequate repair. Mutations were observed 2 - 3 weeks post-irradiation (Páček et al., 2001). Moreover, increases in mutation densities were found in specific genomic regions of cancer samples (namely promoter DNase I-hypersensitive sites (DHS) and 100 bp upstream of transcription start sites (TSS)) that were also found to have decreased DNA repair rates attributable to inadequate nucleotide excision repair (NER) (Perera et al., 2016).

Interestingly, mutation rates have been shown to increase as the required DNA repair becomes more complex. Upon completion of DSB repair in response to radiation and treatment with restriction enzymes, more mutations were found in cases where the ends were non-complementary and thus required more complex DNA repair (1 - 4% error-free) relative to cases where ends were complementary (34 - 38% error-free) (Smith et al., 2001).

Temporal Concordance

There is evidence in the literature suggesting a time concordance between the initiation of DNA repair and the occurrence of mutations. For simple ligation events, mutations were not evident until 12 - 24 hours, whereas DSB repair was evident at 6 -12 hours. For complex ligation events, however, mutations and DSB repair were both evident at 12 - 24 hours. As the relative percent of DNA repair increased over time, the corresponding percent of error-free rejoining decreased over time in both ligation cases, suggesting that overall DNA repair fidelity decreases with time ((Smith et al., 2001).

Essentiality

There is evidence from knock-out/knock-down studies suggesting that there is a strong relationship between the adequacy of DNA repair and mutation frequency. In all examined cases, deficiencies in proteins involved in DNA repair resulted in altered mutation frequencies relative to wild-type cases. There were significant decreases in the frequency and accuracy of DNA repair in cell lines deficient in *LIG4* (Smith et al., 2003) and *Ku80* (Feldmann et al., 2000); rescue experiments performed with these two cell lines further confirmed that inadequate DNA repair was the cause of the observed decreases in repair frequency and accuracy (Feldmann et al., 2000; Smith et al., 2003). In primary Nibrin-deficient mouse fibroblasts, there was increased spontaneous DNA damage relative to wild-type controls, suggestive of inadequate DNA repair. Using the corresponding Nibrin-deficient and wild-type mice, in vivo mutation frequencies were also found to be elevated in the Nibrin-deficient animals (Wessendorf et al., 2014). Furthermore, mutation densities were differentially affected in specific genomic regions in cancer patients depending on their *XPC* status. Specifically, mutation frequencies were increased in *XPC*-wild-type patients at DHS promoters and 100 bp upstream of TSS relative to cancer patients lacking functional *XPC* (Perera et al., 2016). Lastly, in a study using WKT1 cells with less repair capacity, radiation exposure induced four times more mutations in these cells than in TK6 cell, which had a normal repair capacity (Amundson and Chen, 1996).

Uncertainties and Inconsistencies

Repair of alkylated DNA

There were no inconsistencies in the empirical data reviewed or in the literature relating to biological plausibility. Much of the support for this KER comes predominantly from data in somatic cells and in prokaryotic organisms. We note that all of the data in germ cells used in this KER are produced exclusively from ENU exposure. Data on other chemicals are required. We consider the overall weight of evidence of this KER to be strong because of the obvious biological plausibility of the KER, and documented temporal association and incidence concordance based on studies over-expressing and repressing DNA repair in somatic cells.

Repair of oxidative lesions

- Thresholded concentration-response curve of mutation frequency was observed in AHH-1 human lymphoblastoid cells after treatment with pro-oxidants (H₂O₂ and KBrO₂) known to cause oxidative DNA damage (Seager et al., 2012), suggesting that cells are able to tolerate low levels of DNA damage using basal repair. However, increase in 8-oxo-dG lesions and up-regulation of DNA repair proteins were not observed under the same experimental condition.
- Mutagenicity of oxidative DNA lesions other than 8-oxo-dG, such as FaPydG and thymidine glycol, has not been as extensively studied and there are mixed results regarding the mutagenic outcome of these lesions.

Overall

- Mutation induction is stochastic, spontaneous, and dependent on the cell type as well as the individual's capability to repair efficiently (NRC, 1990; Pouget & Mather, 2001).

Quantitative Understanding of the Linkage

Thresholds for mutagenicity indicate that the response at low doses is modulated by the DNA repair machinery, which is effectively able to remove alkylated DNA at low doses [Gocke and Muller 2009; Lutz and Lutz 2009; Pozniak et al. 2009]. Kinetics of DNA repair saturation in somatic cells is described in Muller et al. [Muller et al. 2009].

For O-methyl adducts, once the primary repair process is saturated, in vitro data suggest that misreplication occurs almost every time a polymerase encounters a methylated guanine [Ellison et al. 1989; Singer et al. 1989]; however, it should be noted that this process can be modulated by flanking sequence. This conversion of adducts to mutations also appears to be reduced substantially in vivo [Ellison et al. 1989]. The probability of mutation will also depend on the type of adduct (e.g., O-alkyl adducts are more mutagenic than N-alkyl adducts; larger alkyl groups are generally more mutagenic, etc.). Overall, a substantive number of factors must be considered in developing a quantitative model.

Inadequate repair of oxidative lesions

The relationship between the quantity/activity of repair enzymes such as OGG1 in the cell and the quantity of oxidative lesions need to be better understood to define a threshold on the quantity of oxidative lesions exceeding basal repair capacity. Moreover, the proportion of oxidative lesions formed that lead to mutation versus strand breaks is not clearly understood.

Mutations resulting from oxidative DNA damage can occur via replicative polymerases and translesion synthesis (TLS) polymerases during replication, and during attempted repair. However, an in vitro study on TLS in yeast has shown that bypass of 8-oxo-dG by TLS polymerases during replication is approximately 94-95% accurate. Therefore, the mutagenicity of 8-oxo-dG and other oxidative lesions may depend on their abundance, not on a single lesion (Rodriguez et al., 2013). Applicability of this observation in mammalian cells needs further investigation. Information on the accuracy of 8-oxo-dG bypass in mammalian cells is limited.

The most notable example of mutation arising from inadequate repair of DNA oxidation is G to T transversion due to 8-oxo-dG lesions. Previous studies have demonstrated higher mutation frequency of this lesion compared to other oxidative lesions; for example, Tan et al. (1999) compared the mutation rate of 8-oxo-dG and 8-oxo-dA in COS-7 monkey kidney cells and reported that under similar conditions, 8-oxo-dG was observed to be four times more likely to cause base substitution (Tan et al., 1999).

Inadequate Repair of DSB

Quantitative understanding of this linkage is derived from the studies that examined DSB misrepair rates or mutation rates in response to a radiation stressor. In general, combining results from these studies suggests that increased mutations can be predicted when DNA repair is inadequate. At a radiation dose of 10 Gy, the rate of DSB misrepair was found to be approximately 10 - 15% (Lobrich et al., 2000); this rate increased to 50 - 60% at a radiation exposure of 80 Gy (Kuhne et al., 2000; Lobrich et al., 2000; McMahon et al., 2016). For mutation rates in response to radiation across a variety of models and radiation doses, please refer to the example table below.

Reference	Summary
Matuo et	Yeast cells (saccharomyces cerevisiae) exposed to high LET carbon ions (25 keV/um) and low LET carbon ions (13 keV/um) between 0-200 Gy induces a

al., 2018	24-fold increase overbaseline of mutations (high LET) and 11-fold increase over baseline mutations (low LET).
Nagashima et al., 2018	Hamster cells (GM06318-10) exposed to x-rays in the 0-1 Gy. Response of 19.0 ± 6.1 mutants per 10 ⁹ survivors.
Albertini et al., 1997	T-lymphocytes isolated from human peripheral blood exposed to low LET gamma-rays (0.5-5 Gy) and high LET radon gas (0-1 Gy). Response of 7.0x10 ⁻⁶ mutants/Gy (Gamma-rays 0-2 Gy), 54x10 ⁻⁶ mutants/Gy (Gamma-rays 2-4 Gy) and 63x10 ⁻⁶ mutants/Gy (0-1 Gy).
Dubrova et al., 2002	Observation of paternal ESTR mutation rates in CBAH mice following exposure to acute low LET X-rays (0-1 Gy), chronic low LET gamma-rays (0-1 Gy) and chronic high LET neutrons (0-0.5 Gy). Modelled response of y = mx + C, values of (m,C): X-rays: (0.338, 0.111), Gamma-rays: (0.373±0.082, 0.110), Neutrons: (1.135±0.202, 0.136).
McMahon et al., 2016	Study of HPRT gene in Chinese hamster cells following exposure to radiation of 1-6 Gy. Observation of 0.2 mutations in HPRT gene per 10 ⁴ cells and 0.1 point mutations per 10 ⁴ cells (1 Gy). At 6 Gy, observation of 1.5 mutations in the HPRT gene per 10 ⁴ cells and 0.4 point mutations per 10 ⁴ cells.

Response-response relationship

Inadequate Repair of DSB

There is evidence of a response-response relationship between inadequate DNA repair and increased frequency of mutations. When exposed to a radiation stressor, there was a positive relationship between the radiation dose and the DSB misrepair rate, and between the mutation rate and the radiation dose (McMahon et al., 2016). Similarly, there was a negative correlation found between NER and the mutation densities at specific genomic regions in cancer patients. Specifically, inadequate NER resulted in more mutations in the promoter DHS and the TSS, but normal NER at DHS flanking regions resulted in fewer mutations (Perera et al., 2016).

Time-scale

Inadequate Repair of DSB

Two studies were used to provide data regarding the time scale of DNA repair and the appearance of mutations. In a study using plants, DNA damage was evident immediately following radiation with 30 Gy of radiation; 50% of repairs were complete by 51.7 minutes, 80% by 4 hours, and repair was completed by 24 hours post-irradiation. Although no mutational analysis was performed during the period of repair, irradiated plants were found to have increased mutations when they were examined 2 - 3 weeks later (Pláček et al., 2001). Both DNA repair and mutation frequency were examined at the same time in a study comparing simple and complex ligation of linearized plasmids. In this study, repaired plasmids were first detected between 6 - 12 hours for simple ligation events and between 12 - 24 hours for more complex ligation events; this first period was when the most error-free rejoining occurred in both cases. After this initial period of repair until its completion at 48 hr, repair became increasingly more erroneous such that mutations were found in more than half of the repaired plasmids at 48 hr regardless of the type of required ligation (Smith et al., 2001).

Known modulating factors

Not identified.

Known Feedforward/Feedback loops influencing this KER

Not identified.

References

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Relationship: 2609: Increase, Mutations leads to Increase,miRNA levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
mice	Mus sp.	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Female	High

Not specific through any particular life stage or gender.

Key Event Relationship Description

Upstream event: increased, mutations

Downstream event: increased miRNA

Evidence Supporting this KER

Evidences suggest that transcription pathway for miRNAs is regulated in the DNA damage response (DDR). The tumour suppressor p53 is a well-known transcription factor that is activated in response to DNA damage and causes cell growth arrest, promotes apoptosis, inhibits angiogenesis, and mediates DNA repair (Meek et al., 2009). Global miRNA expression investigations identified the miRNA components of p53 transcriptional pathways and demonstrated that a cohort of miRNAs are up-regulated in a p53-dependent manner following DNA damage. The miR-34 family (miR-34a, miR-34b/c) was the first transcriptional target of p53 to be discovered. p53 directly transactivates miR-15a/16-1, miR-29, miR-107, miR-145, miR-192, miR-194, miR-215, and miR-605 in addition to the miR-34 family.

Biological Plausibility

Various transcription factors regulate miRNA expression. The p53 protein also functions as a transcriptional repressor by binding to miRNA promoters and preventing the recruitment of transcriptional activators. For example, p53 prevents the TATA-binding protein from binding to the TAATA site in the promoter of the miR-17-92 cluster gene, suppressing transcription. Under hypoxic conditions, the miR-17-92 cluster is suppressed by a p53-dependent mechanism, making cells more susceptible to hypoxia-induced death (Yan et al., 2009). Cells can amplify the p53 signal by fine-tuning the p53 signalling pathway and the miRNA network, which improves cell sensitivity to external signals.

Other DNA damage-responsive transcription factors, such as NF- κ B, E2F, and Myc, are also involved in miRNA transcription regulation. Both E2F and Myc promote miR-17-92 cluster transcription, which reduces E2F and Myc expression, establishing an autoregulatory negative feedback loop. Furthermore, Myc-induced miRNAs have an impact on cell proliferation and cell fate in Myc-mediated cells (Kim et al., 2010). Myc-induced miR-20a, for example, targets cdkn1a, a gene that encodes a negative regulator of cell-cycle progression, while Myc-induced miR-221 and miR-222 target the CDKN1b and CDKN1c genes, respectively, that trigger cell-cycle arrest. Little is currently known about how miRNA gene expression is transcriptionally regulated due to a lack of basic information about miRNA gene structure. Global prediction and verification of promoter regions of miRNA genes would allow us to further explore the functional interaction of transcriptional machinery and epigenetic miRNA regulation. p53 regulates miRNA maturation not only during transcription but also during processing of initial miRNA transcripts, resulting in crosstalk between the p53 network and the DDR's miRNA biogenesis machinery. Many p53-regulated miRNAs target proteins in the DDR, such as cell cycle progression and apoptosis, to affect the DDR.

Empirical Evidence

There are findings that strongly link the different elements of DNA damage and repair events to the expression of miRNA.

- Zhang and coworkers examined genome-wide mature miRNA expression in Atm^{+/+} and Atm^{-/-} littermate mouse embryonic fibroblasts to see how miRNAs are regulated in the DNA damage response (MEFs) (Zhang et al., 2011).
- MEFs were given neocarcinostatin (NCS), a radiomimetic medication that causes DSBs (Ziv et al., 2006). Mouse miRNA microarray analysis was used to determine miRNA expression profile in each sample, which was done at several time points (0–24 hr). As many as 71 distinct miRNAs were found to be considerably (2-fold) upregulated in the NCS-treated Atm^{+/+} MEFs, but not in the corresponding Atm^{-/-} MEFs, implying that DNA damage stress causes broad-spectrum changes in miRNA expression.
- The functioning of the Atm gene is required for the induction of these miRNAs. The DNA damage induction of these miRNAs was entirely eliminated when ATM was knocked out of the Atm^{-/-} MEFs, implying that ATM is a critical regulator of KSRP activity in miRNA synthesis.
- To see if DNA damage enhances the transcription of these miRNAs, researchers used quantitative reverse-transcriptase PCR (RT-PCR) using primer sets built particularly for pri-miRNAs to look at the expression levels of primary miRNA transcripts (pri-miRNAs) in both Atm^{+/+} and Atm^{-/-} MEFs. Regardless of ATM status, these pri-miRNAs did not show any significant induction or reduction (50 percent change) in transcription levels during the DNA damage response.
- The study used a pair of human fibroblast cell lines with proficient (GM0637) or deficient (GM9607) ATM to assess the levels of six representative mature miRNAs that were randomly selected out of the pool for both ATM- and KSRP-induced miRNAs to confirm that DNA damage-mediated miRNA induction in MEFs was not species specific.
- Following NCS treatment, the levels of these miRNAs increased dramatically in ATM-proficient cells but not in ATM-deficient cells, matching the findings from MEFs. DNA damage was not induced by the control miR-218, which is not regulated by ATM or KSRP.
- After DNA damage, -KSRP was necessary for miRNA induction. When KSRP was knocked out, the induction of these miRNAs was significantly reduced, demonstrating a functional connection between KSRP and ATM in miRNA synthesis.

- According to Wan et al., regulatory RNA-binding proteins in the Drosha and Dicer complexes, such as DDX5 and KSRP, drive posttranscriptional processing of primary and precursor miRNAs after DNA damage. The findings show that nuclear export of pre-miRNAs is increased in an ATM-dependent manner after DNA damage. The ATM-activated AKT kinase phosphorylates Nup153, a main component of the nucleopore, resulting in enhanced interaction between Nup153 and Exportin-5 (XPO5) and increased nuclear export of pre-miRNAs. These findings demonstrate that DNA damage signalling is important for miRNA transport and maturation.

- To test the DNA-damage induction of miRNAs in human cells, researchers looked at mature miRNA expression in human fibroblast GM0637 cells treated with the radiomimetic drug neocarcinostatin (NCS) in the presence or absence of the ATM inhibitor KU55933.

- In agreement with previous reports showing that ATM-activated p53 and KSRP promote miRNA expression (Suzuki et al., 2009; Zhang et al., 2011), the study found 61 p53-dependent miRNAs and 29 KSRP-dependent miRNAs among the ATM-induced miRNAs.
- The study also examined the levels of different forms of miRNAs (pri- miRNAs, pre-miRNAs, and mature miRNAs) selected from the ATM-induced miRNAs, including KSRP-dependent miRNAs (let-7a, 15a, 15b, 16, 125b, 21, 27b, 98, and 199a), p53- dependent miRNAs (34a), and KSRP/p53-independent miRNAs (181a, 382, and 338).
- As a control, miR-218, which is unaffected by DNA damage, was also included in the examination. With the exception of miR-34a, which is known to be transactivated by p53, there were no significant increases in expression of primary transcripts for these miRNAs following DNA damage. -These results suggest that DNA damage may promote posttranscriptional maturation of the miRNAs.

- Neither KSRP nor p53 were required for the expression of miR-181a, miR-382, or miR-338. Stable knockdown of KSRP or p53 could not prevent their induction following DNA damage, but knockdown of ATM did, indicating that the increased miRNAs in the DDR are accounted for by another ATM-dependent mechanism. The miR-34a and miR-21 controls were reliant on p53 and KSRP, respectively.

- These findings imply that DNA damage may increase miRNA maturation after transcription. Neither KSRP nor p53 were required for the expression of miR-181a, miR-382, or miR-338. Stable knockdown of KSRP or p53 could not prevent their induction following DNA damage, but knockdown of ATM did, indicating that the increased miRNAs in the DDR are accounted for by another ATM-dependent mechanism. As previously observed (He et al., 2007; Trabucchi et al., 2009), the controls miR-34a and miR-21 were reliant on p53 and KSRP, respectively.

- A group of miRNAs is induced in an ATM-dependent way after DNA damage. Prior to NCS (500 ng/ml) treatment, human fibroblast GM0637 (ATM-proficient) cells were pretreated with ATM inhibitor KU55933 (10 mM) or DMSO. For microarray analysis, cells were taken 4 hours after NCS treatment.

- There was an increase in miRNA levels as well as a drop in miRNA levels.
- The miRNA expression profile from GM0637 cells treated with DMSO or ATM inhibitor was used to identify ATM-dependent (ATM-IN/Ctrl 0.67) and ATM-independent (ATM-IN/Ctrl > 0.67) miRNAs.
- Human Mammary Epithelial progenitor Cells (HMEpC) and Human Small Airway Epithelial progenitor Cells (HSAEpC) were cultivated. Cells were cultivated to population doubling 5 before being aliquoted and frozen in Promocell culture media, which contained 10% DMSO and 5% Human Serum Albumin (HSA, Octabline). All tests used cells cultivated from a freshly thawed vial and expanded until population doubling was reached. Up to 16 population doublings, the HMEpC/HSAEpC maintained their proliferation characteristics (Jaarsveld et al., 2014)

- As previously stated, miRNA RT-qPCR expression analysis was performed on 8 normal breast tissue samples and 84 breast tumour tissue samples utilising microfluidic cards (A&B TLDA arrays, Applied Biosystems) (Van der Auwera et al., 2010).
- MiRNA microarray analysis was carried out on i.untreated and cisplatin/IR treated HMEpC and HSAEpC cells (4 replicates for each condition), (ii) 18 lung tumour tissue samples and 14 adjacent 'normal' lung tissue samples (representing NSCLC subtypes), and (iii) 52 breast cancer cell lines (Riaz et al., 2013) and 12 lung cancer cell lines. RNA Bee was used to isolate total —RNA (Bio- Connect, the Netherlands). One mg of RNA was hybridised with an Exiqon LNATM-based probeset (versions 10 and 7 for cell lines and lung tissue, respectively), and spotted in duplicate on Nexterion E slides (Pothof et al., 2009).

- The severity of DNA damage determines the outcome of DDR signalling, i.e. repair and survival or apoptosis/senescence. Since there is evidence in the literature that all these branches within the DDR can be defective in cancer, conditions were established to identify miRNAs that are regulated upon DNA damage in general, independent of biological outcome.
- Two genotoxic therapeutic agents (cisplatin and IR) were used for which a dose was determined that allows for cellular recovery after DNA damage and a dose that induces primarily cell death or senescence.
- To define the conditions for recovery clonal survival assay was used, which determines the capacity of individual cells to recover and form colonies after DNA damage.
- 0.25 mM cisplatin or 1.5 Gy IR were chosen, both resulting in a 50% reduction of HMEpC colony formation , thus 50% of all initially damaged cells can still grow out into a colony within 10 days. --As expected, these conditions activate the DDR and induce cell cycle arrest
- Following that, a higher dose at which the cells suffer apoptosis following cisplatin treatment was determined using an MTT assay. HMEpC viability was reduced by 50% after 48 hours of treatment with 15 mM cisplatin, which was accompanied by lower cellular PARP1 levels, an apoptosis marker. It's worth noting that at this concentration, all cells will eventually die. In contrast to cisplatin, a high dose of IR caused cellular senescence rather than apoptosis, which has no effect on cellular viability as assessed by the MTT experiment.
- HMEpCs were treated with low and high doses of cisplatin and IR to characterise the miRNA response to DNA damage. Total RNA was isolated 6 hours, 12 hours, and 24 hours after the commencement of treatment for each genotoxic treatment and dose based on miRNA kinetics after DNA damage treatment (Pothof et al., 2009; Zhang et al., 2011). MiRNA profiling was carried out on 725 human miRNAs utilising miRNA arrays with Locked Nucleic Acid-based capture probes. Before normalisation, the reproducibility between biological replicates (n 14 4) was examined, revealing that the correlation between replicates was higher than that between non-replicates. After normalisation, condition-specific regulation of miRNAs was shown to be dependent on the kind of genotoxic stress and dose, as well as miRNAs that showed oppo-site regulation after IR and cisplatin treatment.
- General miRNA responders to DNA damage, i.e. significantly regulated miRNAs across all genotoxic conditions per time point were focused. Several general DDR miRNAs were identified and most were regulated at 6 h and 12 h after treatment , which is in agreement with published miRNA expression kinetics after DNA damage (Pothof et al., 2009;). In conclusion, the study could identify several miRNAs that can be characterized as general responders to genotoxic cancer treatments in HMEpCs.

Uncertainties and Inconsistencies

In response to stressors like as ionising radiation, miRNAs are differently regulated. When exposed to IR, miRNA expression is frequently disrupted. Some miRNAs are induced by IR, while others are suppressed, a decision that is likely based on the target genes implicated. This figure summarises the miRNAs mentioned in this review whose expression changes in response to IR. Lists of miRNAs whose induction or repression has been detected are on the left and right, respectively. MiRNAs are in the middle, and both induction and repression have been found in many cell types. The centre contains the biggest group of miRNAs, demonstrating how diverse the miRNA profile can be from one cell type to the next. Bold miRNAs play a role in several parts of the DDR.

Induced miRNAs were reported by some studies (Cha et al.,2009;Chaudhary et al 201; Chaudhary et al 2012; Chaudhary et al 2013;Kwon et al.,2013;Mueller et al.,2013;Shin et al.,2009;Sokolov et al.,2012;Wagner et al.,2010),whereas repressed miRNAs are observed in some(Cha et al., 2009,Chaudhari et al.,2010).Both induction and repression of some miRNA were seen in different cell types and results are inconclusive (Cha et al.,2009;Chaudhary et al 201; Chaudhary et al 2012; Chaudhary et al 2013;Kwon et al.,2013;Mueller et al.,2013;Shin et al.,2009;Sokolov et al.,2012;Wagner et al.,2010; Kraemer et al., 2011; Moskwa et al.,2011;Niemoeller et al.,2011;Sokolov et al., 2012;Wagner et al.,2010). This inconsistency could be due to different doses of stressor.

DNA damage response influences miRNA expression, at the same time miRNA can also influence DDR, cell cycle etc.The miR-34 family produces a cell-cycle arrest in the G1 phase and slows cell-cycle progression by targeting multiple cell cycle regulators when ectopically produced, implying tumor-suppressing potential. The miR-34 family, for example, specifically targets and inhibits cyclin-dependent kinase 4 (CDK4), CDK6, E2F3, Myc, and NMYC (Chang et al.,2007,He et al., 2007).

MiRNA expression can be influenced by DNA damage and mutation, but miRNA can also regulate DNA damage response and cell cycle.By suppressing the transcripts of numerous genes that govern cell-cycle checkpoints or metabolism, these p53-induced miRNAs contribute to cell-cycle arrest (Su et al.,2010;Georges et al.,2008;Hermeking et al.,2012;Klein et al., 2010; Liu et al.,2011; Suh et al.,2011). Wip1 phosphatase, a master inhibitor in the DDR that inhibits the activation and stability of p53, is targeted and repressed by miR-16 and miR-29, resulting in p53 induction (Ugalde et al.,2011; Zhang et al.,2010). Cellcycle arrest is induced by ectopic expression of miR-192/215, which targets a number of genes that regulate the G1/S and G2/M checkpoints (Bulavin et al.,2004).

The oncogene c-Myc is directly targeted by miR-145, implying that p53 suppresses cMyc activities through regulating miRNA expression (Sachdeva et al.,2009, Suh et al.,2011). p53-induced miRNAs, interestingly, influence p53 activity in a positive feedback loop (Han et al.,2012, Hermeking et al.,2012). SIRT1 acetylation and activation are increased when miR-34 inhibits it (Yamakuchi et al., 2008). Mdm2 expression is directly inhibited by miR-192, miR-194, miR-215, and MiR-605, while Wip1 is inhibited by miR-29, resulting in higher p53 levels and activity. (Braun et al.,2008, Pichiorni et al.,2010, Xiao et al., 2011).

Quantitative Understanding of the Linkage

The below table gives the evidence for DNA damage responses influencing the expression of miRNA as well as miRNA expression influencing DNA damage response.

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	Western blotting,clonal survival assay, FACs(Jaarsveld et al., 2014)	YEs	Strong	Yes	Yes	Yes
Mice	Free radic CyQuant cell Proliferation assay (Abdelfattah et al.,2018)	Yes	Strong	Yes	Yes	Yes
	RNA sequence analysis, Immuno staining, immunoblotting, Flowcytometry, COMET assay, qRT PCR(Liu et al., 2017)	Yes	Strong	Yes	Yes	Yes
	Microarray (Zhang et al.,2011)	Yes	Strong	Yes	Yes	Yes
	qRT-PCR, RIP assay, Immunogold EM(Wan et al.,2013)	Yes				
Canine	micro array(Bulkowska et al., 2017)	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Activity of pri-miR-218, pri-miR-16-1, pri-miR-21, and pri- miR-199a had significantly increased binding with Drosha (2.5- to 3.2-fold) after DNA damage (Zhang et al.,2011)

Time-scale

It has been noted that, within hours of DNA damage,miRNA expression were induced(Wan et al.,2013).

Known modulating factors

miRNA expression profiles are influenced by a variety of DNA damaging stressors. Pothof et al. were the first to notice differences in miRNA expression in cell-cycle checkpoints and DNA repair in UV-treated cells (Pothof et al., 2009). Other DNA damaging agents, such as cisplatin, doxorubicin, IR, and NCS, were used to examine miRNA expression profiles in cells (Galluzzi et al., 2010, Saleh et al.,2011;Suzuki et al.,2009). Different levels of DNA damage appear to activate different groups of miRNAs, implying that miRNAs regulate the DDR through a mechanism that is dependent on the type and severity of the DNA damage.

Known Feedforward/Feedback loops influencing this KER

Not specific ones available.

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Relationship: 2610: Increase miRNA levels leads to Decrease SIRT1(sirtuin 1) levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	High

The key event relationship was observed in humans, animals irrespective of gender and life stage specificity.

Key Event Relationship Description

Upstream event: Increased, miRNA

Downstream event: SIRT1, Reduced

Evidence Supporting this KER

Biological Plausibility

There are several signaling pathways that establishing the role of increased miRNA expression in downregulating SIRT 1 gene few of which are listed as follows;

Butyrate-miR-22-SIRT1

Butyrate, a short-chain fatty acid, is produced by the intestinal microbiome via anaerobic fermentation and is subsequently absorbed by the hepatocytes (Besten et al., 2013). Butyrate has been demonstrated to cause apoptosis and reduce carcinogenesis in a variety of cancers (Tailor et al., 2014; Rahmani et al., 2002). Although butyrate has been shown to suppress SIRT1 gene expression in various cancers, this has yet to be proven in hepatocellular carcinoma (HCC) (Iglesias et al., 2007). In HCC, miR-22 was found to be downregulated, and its low levels aided carcinogenesis (Zhang et al., 2010). The Huh7 cells' in vitro proliferation was decreased by miR-22 expression, which activated apoptosis. In Huh7 cells, on the other hand, SIRT1 expression was high, which enhanced the expression of antioxidants such as superoxide dismutase (SOD), allowing cell growth to continue (Chen et al., 2012). Butyrate upregulated miR-22 in Huh7 cells, which binds directly to the 3'UTR region of SIRT1 and suppresses its expression; this decreased SOD function and increased ROS generation, increasing caspase 3 and cytochrome c activity, and encouraging apoptosis (Pant et al., 2017). Furthermore, by downregulating SIRT1, miR22 increased PTEN and gsk-3 expression and downregulated β catenin and p-akt expression and thus may promote apoptosis and decrease HCC proliferation (Pant et al., 2017).

Notch3-SIRT1-LSD1-SOX2 Signaling Pathway

Lysine demethylase 1 (LSD1) is an epigenetic regulator responsible for demethylating various histones and controls the pluripotency of stem cells (Adamo et al., 2011; Whyte et al., 2012; Thambyrajah et al., 2016).

In comparison to normal hepatic parenchyma, HCC cells overexpress LSD1. Furthermore, LSD1 is highly expressed in LCSCs, where it regulates SOX2 gene transcription, promotes self-renewal and carcinogenesis, and is linked to a poor patient prognosis (Liu et al., 2018). LSD1 demethylated the SOX2 promoter, increasing its expression and improving LCSC stemness in a similar way to SIRT1 via DNMT3A. Acetylation suppresses LSD1's enzymatic activity and promotes its breakdown via UPP. SIRT1 enhanced the stability of LSD1 by deacetylating it. Notch signalling is essential for cell survival and proliferation (Bouras et al., 2008). Notch receptors are overexpressed in most HCCs,

and their ligand expression has been linked to aggressive tumour characteristics (Tschaharganeh et al., 2013). The Wnt/-catenin pathway was activated by Notch, which enhanced HCC growth and metastasis (Wang et al.,2016; Wu et al.,2017). Notch signalling has also been demonstrated to increase CSC self-renewal. SIRT1 expression was enhanced by Notch3 signalling, which also promoted LSD1 deacetylation and activated LSD1 which consequently promotes LCSC self-renewal. The Notch3- dependent pathway was crucial for LCSC self-renewal and in vivo tumor dissemination.

MiR-133b is a tumour suppressor that has been found to be significantly decreased in a variety of malignancies (Hu et al.,2010). When compared to paired neighbouring normal tissue, miR-133b expression was shown to be lower in the majority of HCC samples (El-Halawany et al.,2015). Furthermore, overexpression of miR-133b in HepG2 cells inhibited HCC cell growth and invasion while promoting apoptosis (Tian et al.,2016). In nude mice with orthotopic HepG2 cell tumours, increase of miR-133b also reduced tumour growth. In human HCC cells, miR-133b targets SIRT1 and has an adverse relationship with it. Increased miR-133b expression significantly reduced SIRT1 mRNA and protein expression. Overall, miR-133b appears to have an anti-cancer effect in HCC cells through suppressing SIRT1 expression.

In Huh7 and HepG2 cell lines, inhibiting the SIRT1–SREBP pathway lowered proliferation and DNA synthesis, reduced lipid anabolism, and repressed tumorigenesis (Zhang et al.,2014).

MiR-486 inhibits HCC invasion and tumorigenicity by directly targeting and suppressing SIRT1 expression. This reduced the tumorigenic and chemo-resistant features of LCSCs and inhibited HCC invasion and tumorigenicity (Yan et al.,2019).

The miR-29 family inhibits tumour growth by targeting Mcl-1 and Bcl-2. MiR-29c inhibits hepatocytic SIRT1 and so has tumor-suppressing properties. Ectopic miR-29c expression suppressed cell growth by lowering SIRT1 expression. In hepatocytes, miR-29c directly targets and inhibits SIRT1 mRNA translation (Bae et al., 2014).

SIRT1 was downregulated at the mRNA and protein levels when miR-138 expression was increased. MiR-138 binds to the SIRT1 gene's 3'UTR unique complimentary site and inhibits SIRT1 expression directly, preventing HCC proliferation, migration, and invasion (Luo et al.,2017).When compared to the normal hepatic cell line L02, SIRT1 is overexpressed, while miR-138 levels are lowered in HepG2, SMMC7721, Bel7404, and HCCM3

Empirical Evidence

- In Jiang et al's study, the cellular function and molecular mechanism of miR2045p in hepatocellular cancer were investigated (HCC)(Jiang et al.,2016). Real-time reverse transcription polymerase chain reaction was used to analyze at SIRT1 mRNA and miR2045p. Western blotting was used to determine SIRT1 protein levels. To confirm colony formation, a cell proliferation assay was used. The invasion experiment was carried out using a transwell technique.

-Overexpression of miR2045p in human HCC cell lines (BEL7405 and QGY7701) resulted in cell death, increased apoptosis, and increased drug sensitivity. SIRT1 levels were inversely associated to miR2045p levels and were overexpressed in human HCC tissues. These findings suggest that miR204 5p and SIRT1 may play a role in the progression of HCC.

-Introducing miR20405p into BEL7405 and QGY7701 cells lowered SIRT1 mRNA and protein expression. These findings imply that in HCC cells, SIRT1 is a direct target of miR2045.

-It was also found that SIRT1 was negatively related to miR-204-5p expression in HCC tissues.

- Using real-time PCR and western blot assays, Luo et al discovered that miR-138 expression was low while sirtuin type 1 (Sirt1) mRNA expression was high in hepatocellular carcinoma tissues and cell lines, and that miR-138 functions were achieved by targeting Sirt1 using luciferase reporter gene vector and RNA immunoprecipitation assays(Luo et al.,2017).

-Using CCK-8 and BrdU tests, overexpression of miR-138 reduced Sirt1 expression and hindered cell growth.

-Forced production of Sirt1 in cells could partially reverse the inhibitory impact of miR-138. The study findings demonstrated that miR-138 plays a critical role in the regulation of hepatocellular carcinoma cell development via the miR-138/Sirt1 axis, and that miR-138 could be an important future target for hepatocellular carcinoma clinical therapy.

- Shen et al showed that downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signalling(Shen et al., 2016).
- MiR-199b expression levels in six CRC cell lines in comparison to NCM460, a normal colorectal cell line. qRT-PCR detection of relative miR-199b expression after transfection with miR-199b mimics and its negative control (NC).After overexpression of miR-199b, the invasive capacity of SW480 and SW620 cells was examined using the Transwell test.The migratory ability of SW620 and SW480 cells was assessed using a wound healing test. Quantification was done by measuring the wound's smallest clearance distance.After overexpression of miR-199b, Western blot examination revealed the expression levels of invasion-related molecules MMP2 and MMP9, the epithelial-mesenchymal transition (EMT) marker E-cadherin, and Vimentin.The expression of miR-199b was shown to be adversely linked with SIRT1 in CRC specimens in the study. The effects of SIRT1 knockdown on biological behaviour were similar to those of miR-199b overexpression.Furthermore, Human Tumor Metastasis PCR Array revealed that KISS1 was one of SIRT1's downstream targets. SIRT1 silencing increased the acetylation of the transcription factor CREB, which increased KISS1 expression. The latter was activated further by attaching to the KISS1 promoter, which resulted in transcription.

-According to the findings, miR-199b modulates the SIRT1/CREB/KISS1 signalling pathway and could be used as a prognostic marker or a novel treatment target for CRC patients.

- A study by Tian et al found that MicroRNA-133b targets Sirt1 and suppresses hepatocellular carcinoma cell progression(Tian et al., 2016).qRT-PCR was used to examine miR-133b expression levels in 37 cases of primary hepatoma carcinoma tissues and their surrounding normal equivalents.Sirt1 is a direct target of miR-133b, and its expression in HCC is inversely linked with that of miR-133b.After transfecting the miR-133b expression plasmid into HepG2 and SMMC7721 cells, Sirt1 expression was elevated, indicating that the effect of miR-133b on HCC cells is dependent on Sirt1 repression. In comparison to the control group of HCC cells pretreated with miR-133b overexpression vector, transfection of the Sirt1 overexpression vector restored Sirt1 protein levels.Restoring Sirt1 partially reverses the effect of up-regulation of miR-113b on HCC cell proliferation, invasion, and apoptosis in vitro.
- In liver cancer, Yan et al discovered that MicroRNA 486 5p acts as a tumour suppressor of proliferation and cancer stem-like cell characteristics by targeting Sirt1(Yan et al.,2019).

-The qPCR analysis revealed that miR 486 decreased Sirt1 expression in HepG2 and PLC cells. Transfection of a miR 486 inhibitor, on the other hand, enhanced Sirt1 mRNA levels.

-The study focused primarily on Sirt1 as a miR 486 target gene. A luciferase assay in 293T cells was used to further investigate the connection between miR 486 and Sirt1 3'UTR. The Dual Luciferase Assay System was used to detect luciferase activity in cells co-transfected with miR 486 mimics or NC using the pGL3-Sirt1-3'UTR vector or pGL3 basic vector. Sirt1-3'UTR reporter firefly luciferase activity was considerably reduced by co-transfection with miR 486 mimics, but not by the pGL3 reporter. Western blot analysis revealed that in the miR 486 overexpression group, Sirt1 was downregulated. Furthermore, IHC labelling revealed that Sirt1 expression was downregulated in tumour tissues produced from miR 486 overexpression cells.

- Zhang et al reported that MicroRNA-22 functions as a tumor suppressor by targeting SIRT1 in renal cell carcinoma (Zhang et al., 2016).

-Real-time quantitative RT-PCR was used to determine the miR-22 expression pattern (qRT-PCR).

-Quantitative real-time PCR results revealed that miR-22 was considerably downregulated in RCC samples compared to non-cancerous tissues, and this was related with tumour stage and lymph node metastasis.In vitro, forced overexpression of miR-22 decreased proliferation, migration, and invasion, as well as promoted cell death, and suppressed tumour growth in vivo, according to a functional investigation.In addition, a luciferase reporter test showed SIRT1 as a direct target of miR-22.

-MiR-22 overexpression suppressed epithelial-mesenchymal transition via activating p53 and its downstream targets p21 and PUMA, as well as the apoptosis markers CASP3 and PARP (EMT). These findings revealed that miR-22 acted as a tumour suppressor in RCC and inhibited RCC growth and metastasis by directly targeting SIRT1, implying an unique therapeutic role for RCC treatment.

- Astragalus Polysaccharides(APS) inhibits Tumorigenesis and Lipid Metabolism Through the miR-138-5p/SIRT1/SREBP1 Pathway in Prostate Cancer (Guo et al.,2020).APS was discovered to suppress the proliferation and invasion of PCa cells in vitro and in vivo in a dose- and time-dependent manner in the current investigation.Under APS exposure, SIRT1 expression was significantly decreased, according to microarray results.

-SIRT1 knockdown enhances AMPK/SREBP1 signalling and its related target genes considerably.

- Bae et al reported that MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma(Bae et al., 2014).

-In this study, SIRT1 expression in a subset of HCCs was performed.

-The study found that overexpression of SIRT1 increased HCC cell proliferation by inactivating p21Cip1, p27Kip1, and p15INK4B, as well as activating CDK2, CDK6, cyclin D3, and cyclin D1.

-In addition, miRNA expression profiling was used to explore for deregulated miRNAs in HCC, and five miRNAs targeting SIRT1 were shown to be highly downregulated in the disease.

-The liver cancer cell lines SNU-182, HepG2, and Hep3B grew slower when SIRT1 was knocked off.

-miR-29c inhibits SIRT1 mRNA translation in the liver, acting as a tumour suppressor. In the rescue experiment, SIRT1-expressing plasmid (pcDNA3.1- SIRT1-His) or Mock (empty vector, pcDNA3.1-His) were introduced into SNU-182 cell lines in the presence or absence of ectopic miR-29c expression. MiR-29c mimic (miR-29c) and 30 UTR-negative SIRT1 expression plasmids were transfected into cells (pcDNA3.1-SIRT1-His).

-After 48 h of transfection, endogenous SIRT1, not ectopically expressed SIRT1 (SIRT1-His), was suppressed by ectopic miR-29c transfection as found in Western blot analysis .

-Anti-growth effect of ectopic miR-29c was attenuated by co-transfection of SIRT1-expressing plasmid

- In a study by Zhou et al,the miR-34a overexpression vector or scramble control was transfected into human Hep3B and Huh7 cell lines(Zhou et al 2017).

-To determine the impact of miR-34a expression on HCC cell invasion and migration, researchers performed Transwell assays, Matrigel, and wound healing experiments.

-Using quantitative reverse transcription polymerase chain reaction, the expression of miR-34a and the mRNA expression of other related proteins was discovered, and protein levels were determined using western blot analysis.

-MiR-34a expression was considerably downregulated in Hep3B and Huh7 cells compared to the control, however this was reversed by transfection with exogenous miR-34a.

-Overexpression of miR-34a increased the expression of sirtuin 1 while decreasing the amount of acetylate-p53.

- Study by Fu et al concluded that MiRNA-200a induce cell apoptosis in renal cell carcinoma by directly targeting SIRT1(Fu et al.,2018).The expression of miR-200a was found to be considerably lower in renal cell carcinoma

(RCC) specimens and RCC cell lines in the study. In RCC cell lines, restoring miR-200a decreased cell growth, halted cell cycle progression, and accelerated cell death.

-Sirtuin 1 (SIRT1) was identified as one of the downregulated proteins after miR-200a overexpression in 786-O cells using qRT-PCR array technique. SIRT1 was confirmed as a direct target of miR-200a after a second assay using a luciferase reporter system. Furthermore, knocking down SIRT1 with siRNA could partially mimic the effects of miR-200a overexpression.

-Overexpression of truncated SIRT1 (without an endogenous 30'-UTR) on 786-O cells, on the other hand, may rescue the effect of miR-200a overexpression, suggesting that the SIRT1 30'-UTR is preferentially targeted by miR-200a.

-These findings add to the growing body of data for the miR-200a's tumor-suppressive effect in RCC, as well as establishing a novel regulatory mechanism that could play a role in SIRT1 overexpression in RCC.

- Study by Lian et al. performed Quantitative real-time PCR analysis to detect the expression of microRNA-128 (miR-128) in tissues from patients with CRC and CRC cell lines. The effect of miR-128 on Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an anti-tumor medication, caused cytotoxicity against CRC cell lines was assessed using MTT assays (Lian et al.,2018).

-Flow cytometry was used to detect the distribution of death receptor 5 (DR5) and the formation of reactive oxygen species (ROS).Western blot, flow cytometry, and luciferase reporter assays were used to look into the mechanism and pathway of miR-128-induced apoptosis in TRAIL-treated CRC cells.

-MiR-128 expression was found to be downregulated in CRC tumour tissues as well as CRC cell lines in vitro. MiR-128 overexpression made CRC cells more susceptible to TRAIL-induced cytotoxicity through triggering apoptosis. MiR-128 directly targeted sirtuin 1 (SIRT1) in CRC cells, according to bioinformatics, western blot analysis, and luciferase reporter tests. Overexpression of miR128 reduced SIRT1 expression, which increased ROS generation in TRAIL-treated CRC cells.

This increase of ROS subsequently induced DR5 expression, and thus increased TRAIL-induced apoptosis in CRC cells.

- A study by Guan et al confirmed SIRT1 as a direct target of miR-30a by measuring SIRT1 expression in lung cancer cells following overexpression or knockdown of miR-30a and using a luciferase assay (Guan et al.,2017).

-In vitro and in vivo, miR-30a decreased lung cancer cell proliferation, invasion, and increased apoptosis through inhibiting SIRT1.This research discovered a new regulatory axis in which miR-30a and SIRT1 control lung cancer cell proliferation, invasion, and apoptosis, as well as lung carcinogenesis. After overexpression or knockdown of miR-30a, two human lung cancer cell lines (A549 and H1975) were employed to confirm the direct link between miR-30a and SIRT1.Cellular levels of miR-30a were greatly elevated in A549 and H1975 cells transfected with miR-30a mimics and dramatically decreased in cells transfected with miR-30a inhibitor, as expected.As a result, overexpression of miR-30a dramatically decreased SIRT1 protein expression in A549 and H1975 cells, whereas the miR-30a inhibitor considerably boosted SIRT1 protein levels in lung cancer cells. The studies were repeated, and the expression of SIRT1 mRNA after transfection was evaluated to see how much miR-30a influenced SIRT1 expression. The level of SIRT1 mRNA was unaffected by overexpression or knockdown of miR-30a.

- In cells co-transfected with luciferase reporter plasmid and miR-30a mimics, luciferase activity was drastically reduced. Then, to delete the expected miR-30a binding site, point mutations were induced into the SIRT1 3'-UTR binding region. The overexpression or knockdown of miR30a has no effect on this mutant luciferase reporter. This finding revealed that the binding sites play a significant role in miR-30a-SIRT1 mRNA interaction.

- Yang et al suggested Down-Regulation of miR-221 and miR-222 Restrain Prostate Cancer Cell Proliferation and Migration That Is Partly Mediated by Activation of SIRT1(Yang et al.,2014).

- When compared to LNCap cells, PC-3 cells had higher levels of miR-221 and miR-222 expression. The proliferation and migration rates of PC-3 cells dropped after miR-221 or miR-222 expression was inhibited, but the apoptosis rate rose. Furthermore, after cells were transfected with miR-221 or miR-222 inhibitors, SIRT1 protein was up-regulated. In comparison to the controls, cells transfected with siSIRT1 demonstrated greater migration and a lower rate of apoptosis, but no significant influence on cell proliferation. Although there was a negative connection between miR-221 and SIRT1, no direct target relationship was discovered. These findings show that miR-221 and miR-222 are abundant in PC-3 cells. In prostate cancer cells, inhibiting these proteins reduces cell proliferation and migration while increasing apoptosis. Upregulation of SIRT1 may be responsible for these effects.

Uncertainties and Inconsistencies

Not specific.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human tissues	qRT-PCR,Western blotting,Luciferase reporter assay Micro-array	Yes	Strong	Yes	Yes	Yes
Human cell lines	Micro-array, qRT- PCR,Western blotting, Luciferase reporter assay Micro-array	Yes	Strong	Yes	Yes	Yes
Mouse(A1)	qRT-PCR,Western blotting,Luciferase reporter assay,ELISA,cell culture	Yes	Moderate	Yes	Yes	Yes

Response-response relationship

No specific pattern of response response relationship was observed.

Time-scale

In study by Jiang et al, it was shown that miR-204-5p targeting SIRT1 regulates hepatocellular carcinoma progression. The results were noted within 48 hours during the experiment (Jiang et al., 2016).

Known modulating factors

SIRT1 has been found to have a number of endogenous and external regulators. SIRT1 activity is naturally inhibited by the protein encoded by deleted in breast cancer 1 (DBC1). DBC1 forms a tumour suppressor complex with SIRT1, but knocking out DBC1 increases SIRT1 activity, promoting tumorigenesis. The small molecule resveratrol was the first to be discovered to regulate SIRT1 activity and extend life span. After the effect of resveratrol upon SIRT1 was characterized, high throughput screening was used to find other small molecule activators of SIRT.

Known Feedforward/Feedback loops influencing this KER

p53-miR-34a-SIRT1 Signaling Pathway

0404 is a DNA-damaging substance that has no cytotoxic effects on human hepatocytes that aren't malignant. In an in vivo HepG2 HCC model, 0404 caused apoptosis and inhibited proliferation. P53 WT HepG2 cells, on the other hand, were more susceptible to 0404 than p53 mutant Huh7 cell lines (Xia et al.,2017). P53 influences the expression of several miRs. As a result, a large number of miRs target the 3'UTR region of the p53 mRNA. As a result, p53 and miRs could establish a feedback loop (Zhang et al., 2015). The miR-34 family has been identified as the most common p53-induced miRs and is commonly suppressed in diverse malignancies (Xiao et al.,2014, Lou et al.,2015). In HCC cells, miR-34a increased p53 transcription and acetylation while also inducing apoptosis. 0404 enhanced p53 and miR-34a expression, elevated acetylated p53, and downregulated SIRT1 protein expression in HepG2 but not Huh7 cell lines, inhibiting HCC growth (Xiao et al.,2014).

In HCC, the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is highly expressed, promoting development and invasion. MALAT1 stimulates the formation of HCC CSCs by activating the mechanistic target of rapamycin (mTOR) signalling pathway (Malakar et al.,2017;Yuan et al 2016). MiR-204, in contrast to MALAT1, promotes apoptosis by activating p53 and suppressing Bcl-2, an anti-apoptotic protein (Ryan et al.,2012). Cancer stemness and EMT were also suppressed by miR-204, which increased chemosensitivity (Ryan et al.,2012 ;Sacconi et al.,2012). MALAT1 expression, on the other hand, was negatively linked with miR-204 levels. MALAT1 binds to miR-204 and inhibits its expression by binding directly to it (Hou et al., 2017). SIRT1 appears to play a key role in the interaction between MALAT1 and miR-204. SIRT1 is recognised to play a role in HCC EMT, migration, and invasion. MiR-204 specifically targets SIRT1 and silences it (Hou et al.,2017).

However, because SIRT1 and MALAT1 bind to the same miR-204 region, MALAT1 may compete with SIRT1 for miR204 binding, reducing miR-204-induced SIRT1 suppression. Overall, MALAT1 inhibited miR-204 activity, resulting in an elevation in SIRT1, which encouraged HCC migration and invasion (Hou et al., 2017). MALAT1 inhibition reduced the aggressiveness of HCC, making it a possible therapeutic target (Hou et al., 2017).

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Relationship: 2611: Decrease,SIRT1(sirtuin 1) levels leads to Increase activation, Nuclear factor kappa B (NF-κB)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	Moderate	NCBI
mice	Mus sp.	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
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Not Otherwise Specified
Life Stage

Not Specified
Evidence

Sex Applicability

Sex Evidence

Female High

The KER has been noted in human and animal cell lines irrespective of gender or any specific life stage.

Key Event Relationship Description

Upstream event: Decreased, SIRT1

Downstream event: NF-kB activity, Increased

Evidence Supporting this KER

SIRT1 deacetylates NF-kB. In the context of NF-kB, all of the evidence so far points to its signalling being inhibited after SIRT1 deacetylation (Morris, 2012). SIRT1 or SIRT1 activation by resveratrol and other polyphenols, in fact, has been found to reduce inflammatory response by deacetylating and inhibiting NF-kB in both in vitro and in vivo investigations. The essential significance of NF-kB in many cellular processes implicated in inflammation, ageing, cancer, and other diseases makes these findings particularly intriguing.

Biological Plausibility

-The acetylation of many lysines on NF-kB has been identified, which leads to its activation (Kierman et al., 2003). A novel class of deacetylases known as Sirtuins has heightened interest in modulating NF-kB activity. The activation of sirtuins actually inhibits NF-kB.

- According to Yeung et al. SIRT1 can directly interact with and deacetylate the RelA/p65 component of the NF-kB complex (Yeung et al.,2004). Deacetylation of Lys310 decreased the transactivation ability of the RelA/p65 subunit and, as a result, lowered the transcription of NF-kB-dependent genes. Furthermore, deacetylation of Lys310 in the RelA/p65 protein exposed it to methylation at Lys314 and Lys315, resulting in increased ubiquitination and destruction of the protein (Yang et al.,2010). SIRT1 inhibition of NF-kB signalling has been demonstrated in a number of recent studies, and activation of SIRT1 could ameliorate a variety of NF-kB-driven inflammatory and metabolic illnesses (Salminen et al.,2008; Yu et al.,2010; Yao et al.,2012; Xie et al.,2013).

SIRT1 suppresses NF-kB signalling either directly by deacetylating the RelA/p65 subunit or indirectly by triggering repressive transcriptional complexes, which frequently involve heterochromatin formation at NF-kB promoter regions. SIRT1 expression and signalling are both inhibited by NF-kB.

Zhang et al. found that overexpressing RelA/p65 protein increased SIRT1 expression at both the transcriptional and protein levels (36 h treatment), whereas knocking down RelA/p65 expression decreased TNF-induced SIRT1 expression (8 h treatment)(Zhang et al.,2010). They also discovered that the RelA/p65 protein may bind to the SIRT1 promoter's NF-kB motifs. These findings suggest that NF-kB may promote SIRT1 expression. Given that SIRT1 induction appeared to occur much later than NF-kB activation, it appears that this action could represent a feedback response limiting inflammation and eventually generating endotoxin tolerance.

Empirical Evidence

- According to Lu et al, SIRT1 inhibited the growth of gastric cancer through inhibiting the activation of STAT3 and NF-kB (Lu et al.,2014). The goal was to look at SIRT1's regulatory effects on gastric cancer (GC) cells (AGS and MKN-45) as well as the links between SIRT1 and STAT3 and NF-kB activation in GC cells. The SIRT1 activator (resveratrol RSV) was discovered to contribute to the repression of viability and increase of senescence, which was reversed by SIRT1 inhibitor (nicotinamide NA) and SIRT1 depletion using the CCK-8 and SA-B-gal assays, respectively. SIRT1 activation (RSV supplement) reduced not only STAT3 activation, including STAT3 mRNA level, c-myc mRNA level, phosphorylated STAT3 (pSTAT3) proteins, and acetylated STAT3 (acSTAT3) proteins, but also pNF-kB p65 and acNF-kB p65 suppression. The effects of RSV were reversed by NA.
- Furthermore, when STAT3 or NF-kB were knocked down, neither RSV nor NA could affect cellular survival or senescence in MKN-45 cells. Overall, the outcomes of the study revealed that SIRT1 activation could cause GC in vitro to lose viability and senescence. Furthermore, our findings demonstrated that SIRT1 inhibited proliferation in GC cells and was related with deacetylation-mediated suppression of STAT3 and NF-kB protein activation.
- The levels of SIRT1 protein expression in non-small-cell lung cancer (NSCLC) cell lines were examined in a study by Yeung et al.,2004. In comparison to immortalised epithelial human lung NL-20 cells, NSCLC cells exhibit significant quantities of SIRT1 protein, as reported by other researchers (Luo et al, 2001; Vaziri et al, 2001).
- Pharmacological modulators of Sirtuin activity were employed to see if NF-kB transcription was regulated by Sirtuins (Landry et al, 2000; Bedalov et al, 2001; Howitz et al, 2003).

Transient luciferase reporter experiments revealed that cells pretreated with resveratrol had very minimal NF-kB transcription following the presence of TNF α . TNF α -induced NF-kB activity was boosted when cells were pretreated with the Sirtuin inhibitors nicotinamide or splitomicin. NF-kB transcription was also potentiated in cells treated with trichostatin A (TSA), an HDAC class I and class II inhibitor, as expected.

Uncertainties and Inconsistencies

- SIRT1 can inhibit NF-kB signaling directly or indirectly, in turn the NF-kB system suppresses SIRT1-mediated functions by inhibiting the downstream targets of SIRT1. Given that SIRT1 and NF-kB signaling have antagonistic characteristics, these pathways control many of the physiologically relevant metabolic and inflammatory switches required for the maintenance of cellular and organismal homeostasis.
- PGC-1 is a downstream target of the SIRT1/AMPK signalling cascade that promotes oxidative metabolism by promoting mitochondrial biogenesis (Fernandez et al.,2011). In cardiac cells, Alvarez-Guardia et al. found that the RelA/p65 member of the NF-kB complex was constitutively linked to the PGC-1 protein. They also discovered that activating NF-kB after TNF exposure boosted the association between the RelA/p65 and PGC-1 proteins, resulting in an increase in glucose oxidation (Alvarez et al., 2010). These findings show that deacetylation of PGC-1 promotes mitochondrial oxidative respiration, whereas activation of NF-kB signalling inhibits SIRT1/PGC-1 communication and activates aerobic glycolysis. This shift is known as the Warburg effect, which can be seen in cancer cells but also in ageing (Salminen et al., 2010). Overexpression of PGC-1, on the other hand, decreased the transcriptional activity of NF-kB by lowering the phosphorylation of the transactivating RelA/p65 component(Eisele et al.,2013)

Quantitative Understanding of the Linkage

	Method/ reference	measurement	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	qRT-PCR, Luciferase reporter assay	Cell based HDAC assay(Luo et al.,2001)	Yes	Strong	Yes	Yes	Yes
Humans	qRT- PCR,immunohistochemistry (McGlynn et al.,2014)		Yes	Strong	Yes	Yes	Yes
Mouse	qRT-PCR,Southern and northern blotting, reporter gene assay(Paul et al.,2008)		Yes	Low	Yes	Yes	Yes

Response-response relationship

- Studies have been done on pancreatic cancer cells, Joudah et al colleagues investigated the processes and correlations between SIRT1 and NF-kB activation .The results showed that a 1 μ M SIRT1 aptamer might limit NF-kB activation by increasing SIRT1 protein activity(Joudah et al.,2021). According to the findings of SIRT1 aptamer mechanisms, it is possible that SIRT1 aptamer will be used in the treatment of pancreatic cancer in the future.

-To explore the mechanism of SIRT1 aptamer in cell lines, SIRT1 activity was measured in parallel on Aspc-1, BxPc-3, and Capan-2 cell lines under the same conditions. SIRT1 activity was measured in BxPc-3 cell lines using SIRT1 aptamer at 0.25, 0.5, and 1M. Then, using 100M resveratrol (SIRT1 activator control), 100M suramin, and nicotinamide(SIRT1 inhibitor control), assess its activity .

-The results revealed that using SIRT1 aptamer at 1M boosted SIRT1 activity in Capan-2 cells when compared to high concentrations of 100M resveratrol, 100M Suramin, and 100M Nicotinamide.

-The activation of SIRT1 in the Aspc-1 cell line when treated with SIRT1 at 1 μ M was higher than that of 100 μ M resveratrol, Suramin, and Nicotinamide.

-the effect of SIRT1 aptamer on NF-kB activation was determined in nuclear extracts of BxPc-3, Capan-2, and AsPC-1 cell lines using an ELISA-based test to measure the capacity of NF-kB p65 subunit for DNA-binding.

-At 1 μ M, adding a SIRT1 aptamer caused biphasic alterations in NF-kB. At 8 hours, NF-kB binding activity in Bx-PC-3, Capan-2, and AsPC-1 cell lines was reduced by 150 percent, 130 percent, and 130 percent, respectively, compared to control 100 percent. In Bx-PC-3,Capan-2, and AsPC-1 cell lines, the decline was 180 percent, 145 percent, and 140 percent of the control 100 percent, P<0.005 at 16 hours respectively.

Time-scale

The events connected by this KER occur within hours.

Known modulating factors

- NF-kB can be activated by cytokines (TNF-, IL-1), growth factors (EGF), bacterial and viral products (lipopolysaccharide (LPS), dsRNA), UV and ionising radiation, reactive oxygen species (ROS), DNA damage, and oncogenic stress from inside the cells. Almost all stimuli eventually activate a large cytoplasmic protein complex called the inhibitor of κ B (IB) kinase (IKK) complex via a so-called "canonical pathway." The exact composition of this complex is unknown, however it has three fundamental components: IKK1/IKK β , IKK2/IKK α , and NEMO/IKK γ . IB is phosphorylated by the activated IKK complex, which marks it for destruction by the -transducin repeat-containing protein (-TrCP)-dependent E3 ubiquitin ligase-mediated

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proteasomal degradation pathway (Liu et al., 2012;Li et al., 2002). As a result, unbound NF- κ B dimers can go from the cytoplasm to the nucleus, bind to DNA, and control gene transcription.

- SIRT6 is a nuclear sirtuin that regulates the acetylation status and transcriptional activity of HIF1 and NF κ B. SIRT6 deacetylates histone 3 lysine 8 (H3K9) at HIF1 target gene promoters and so acts as a corepressor of HIF1 transcriptional activity. SIRT6 modulation of glucose flow appears to be crucial, as SIRT6 deficiency results in fatal hypoglycemia (Zhong et al., 2010). SIRT6 inhibits NF κ B function through a mechanism that is strikingly similar. SIRT6 also deacetylates H3K9 on the promoters of specific NF κ B target genes, reducing NF κ B's accessibility to these promoters (Kawahara et al., 2009). SIRT6 has a compensating impact in SIRT1 deficient animals, attenuating the enhanced NF κ B activity due to an elevated acetylation state (Schug et al., 2010). Finally, although having different methods, both SIRT1 and SIRT6 are negative regulators of NF κ B activity.
- SIRT2 has been demonstrated to deacetylate the cytoplasmic lysine 310 (K310) of NF κ B subunit p65 (Rothgiesser et al., 2010). SIRT2 suppresses NF κ B activation and transcription of NF κ B target genes in response to TNF stimulation in this way (Rothgiesser et al., 2010). After TNF exposure, SIRT2 silenced cells show higher NF κ B activity and a reduced probability of cell death (Rothgiesser et al., 2010). As a result, SIRT2 in the cytosol and SIRT1 in the nucleus can both deacetylate NF κ B.

Known Feedforward/Feedback loops influencing this KER

- SIRT1 and AMPK have a close interaction in the control of energy metabolism and inflammation as they can promote each other's activity (Ruderman et al., 2010). SIRT1 stimulates AMPK by deacetylating LKB1, which then activates AMPK (Lan et al., 2008), whereas AMPK promotes the synthesis of cellular NAD $^{+}$, which is necessary for SIRT1 activity (Canto et al.,2009). SIRT1 and AMPK have many similar activities in the control of energy metabolism as a result of this positive feedback.
- AMPK appears to be an efficient inhibitor of NF-B signalling and inflammatory reactions, according to new research. This topic was recent discussed in depth (Salminen et al.,2011). In a nutshell, AMPK inhibits RelA/p65 by activating SIRT1. PGC-1 is also phosphorylated by AMPK, which increases its activation (Canto et al.,2009). As a result, PGC-1 can block RelA/p65-mediated NF-B signalling.
- The transcription factor FoxO3a, which is involved in metabolic and immunological homeostasis, was activated by AMPK (Eijkelenboom et al.,2013). Overexpression of FoxO3a decreased NF-B activation in cultured cells, such as after TNF treatment, by suppressing nuclear translocation of the RelA/p65 component. The inhibition of NF-B signalling by FoxO3a was corroborated in a study (Lee et al.,2008) who found that overexpression of FoxO3a caused the production of B-Ras1, an inhibitor of NF-B activation. However, FoxO3a has recently been discovered to activate the NF-B system via BCL10, which is expressed in B lymphocytes (Li et al., 2012).

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Relationship: 2612: Increase activation, Nuclear factor kappa B (NF- κ B) leads to Antagonism, Estrogen receptor

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

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Cell line	Immunoprecipitation, immunofluorescent microscopy, Luciferase reporter assay EMSA,	Yes	Strong	Yes	Yes	Yes
Humans	IHC, Cell viability assay, qRT-PCR, immunohistochemistry	Yes	Strong	Yes	Yes	Yes
Mouse(A1)	EMSA, Autoradiography Immunofluorescent microscopy, Westernblotting	Yes	Strong	Yes	Yes	Yes

Response-response relationship

Differential Sensitivity of ER α and ER β Cells to the NF- κ B inhibitor Go6976. A differential sensitivity to Go6976 by ER α and ER β breast cancer cells was observed (Holloway et al., 2004). The ER α cells were more sensitive and less viable after treatment with this NF- κ B inhibitor. The IC₅₀ (50% killing) by Go6976 was 1 mM for Era of MDA-MB435 and MDA-MB231 breast cancer cells, whereas it was greater than 10 mM for ER α of MCF-7 and T47D or the normal mammary epithelial H16N cells. At 10 mM Go6976, about 80% of the ER α cells were killed, whereas only 15–30% of ER α and normal H16N cells were sensitive to this compound. The relative resistance of the H16N normal human mammary cells indicates a possible high therapeutic index of Go6976 against ER α cancer cells.

This observation is consistent with the previously observed role of NF- κ B as an antiapoptotic agent. FACS analysis demonstrated accumulation of sub-G1 population (67%) in Go6976- treated (48 h at 1 mM) ER α vs. only 10–15% in ER α cells, indicating enhanced apoptotic cell death preferentially of ER α cells caused by this low molecular weight compound.

Time-scale

Key events connected by this KER occur within hours of exposure.

Known modulating factors

Estradiol has been shown to decrease transcriptional activity and expression of NF- κ B in a variety of experimental models (Biswas et al., 2005; Lobanova et al., 2007). Estrogen treatment of MCF-7 or MCF-7/H cells resulted in a significant suppression of NF- κ B activity in both cell lines, according to research. The antiestrogen tamoxifen boosted NF- κ B activity in the cells, indicating that ER plays a key role in NF- κ B down-regulation in both parent and hypoxia-tolerant cells.

-MCF-7/T2H cells were discovered to have a partial tolerance to acute cobalt chloride-induced hypoxia while maintaining their estrogen-independent phenotype. In contrast to the MCF-7/H subline, MCF-7/T2H cells had a non-affected baseline NF- κ B level, indicating that estrogens are responsible for NF- κ B downregulation (Scherbakov et al., 2009).

Known Feedforward/Feedback loops influencing this KER

- Multiple pathways are implicated in the crosstalk between NF- κ B and ER. Through many mechanisms, including collaboration with FOXA1 to strengthen latent ER-binding sites and trigger translation of their synergistic genes, NF- κ B directly interacts with the DNA-binding activity of ER (Franco et al. 2015). Furthermore, NF- κ B affects ER via interacting with its ER co-activator or co-repressor, which changes ER transcriptional activity (Park et al. 2005). Similar to ER, NF- κ B has been reported to have a role as a downstream effector for the growth factor pathway, which is recognized to be involved in both ligand-dependent and non-ligand-dependent ER activation, leading to resistance to a wide range of anti- oestrogen drugs (Zhou et al. 2005a, Sas et al. 2012, Frasor et al. 2015).

-NF- κ B is also involved in the anti-apoptotic pathway and immune surveillance systems, both of which have been linked to endocrine resistance (Hu et al. 2015; Lim et al. 2016). Furthermore, NF- κ B inhibition of ER activity has been observed. The zinc finger repressor B-lymphocyte-induced maturation protein (BLIMP1), which can bind to the ER promoter area and restrict ER transcription, is triggered by the NF κ B subunit RelB. (Wang et al. 2009). Increasing data suggests that NF- κ B plays an important role in the complexities of the endocrine resistance environment in breast cancer.

-NF-KB and ERS1 mutations in breast cancer patients who are resistant to endocrine therapy

TNF needs NF- κ B and FOXA1 to change the breast cancer cell transcriptome by modulating latent ER-binding sites.

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Relationship: 2613: Antagonism, Estrogen receptor leads to EMT

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Mixed	High

Humans and animals with no specific gender or life stage specificity.

Key Event Relationship Description

Upstream event: Decreased, Estrogen receptor activity

Downstream event: EMT, Increased

Evidence Supporting this KER

Estrogen/ERα signaling maintains an epithelial phenotype and suppresses EMT.ERα signaling promotes proliferation and epithelial differentiation and opposes EMT. ERα activated by E2 inhibits TGF-β signaling and cytokine

signaling through Smad and NF- κ B, respectively, both of which promote EMT. EMT-related transcription factors and microRNAs are likewise suppressed by ER α signalling. This anti-EMT stance is thought to be a major component in luminal A breast cancer's low spreading potential and excellent prognosis. ER α signalling, on the other hand, promotes the proliferation and survival of ER α -positive breast cancer cells by increasing cell cycle and anti-apoptotic gene expression. Furthermore, because GATA3 is a marker for luminal progenitor cell development and both GATA3 and FOXA1 are cofactors that affect ER α signalling and activity, ER α signalling interacts with luminal-related transcription factors GATA3 and FOXA1 to promote an epithelial phenotype. These elements work together to enhance cell–cell adhesion, basolateral polarity, and low motility in epithelial tissues.

Biological Plausibility

E2/ER α signalling, in part through transcriptional activation of luminal/epithelial-related transcription factors, promotes the development of mammary epithelia along a luminal/epithelial lineage. GATA3 and ER α both promote each other (Eeckhoutte et al.,2007). In normal breast epithelia, GATA3 is needed for luminal differentiation(Kouros-Mehr et al.,2008) and GATA3 and ER α control many of the same genes (Wilson et al.,2008). In mice, forcing GATA3 expression in mesenchymal breast cancer cells produces mesenchymal–epithelial transition (MET), a reversible mechanism analogous to EMT, and prevents tumour metastasis (Yan et al.,2010). Another ER α -interacting transcription factor, FOXA1, is essential for luminal lineage in mammary epithelia and stimulates ductal development in mice (Bernardo et al.,2010). FOXA1 enhances ER α gene expression by increasing the accessibility of estrogen-response regions for ER α binding (Nakshatri et al., 2009). In breast cancer cells, on the other hand, E2 appears to increase FOXA1 expression. Importantly, ER α , FOXA1, and GATA3 are all positive breast cancer prognostic factors(Nakshatri et al.,2009).

ER α signalling enhances primary lesion formation (and therefore is mitogenic), but it can control the EMT process (and thus is anti-metastatic) up to a point. Signaling pathways that lead to EMT are antagonised by E2/ER α signalling. TGF- β , for example, has been demonstrated to generate EMT in human mammary epithelial cells, and overexpression of the EMT-inducing protein Snail boosted TGF- β signalling and invasiveness while decreasing adhesion and ER α expression in MCF-7 cells (Taylor et al.,2010). TGF- β has an anti-estrogen impact on MCF-7 cells. Smad2/3 and the Smad-selective E3 ubiquitin ligase Smurf create a ternary complex with ER α , which enhances the proteosomal degradation of Smad proteins, according to Ito et al (Ito et al.,2010).

Empirical Evidence

• Ye et al. investigated the impact of ER α overexpression in ER α -negative breast cancer cell lines (MDA-MB-468, MDA-MB-231) or ER α knockdown in ER α -positive cell lines (MCF-7, T47D) on Slug and Snail expression and phenotypes in ER α -positive cell lines (MCF-7, T47D)(Ye et al., 2010). Slug is repressed, E-cadherin is increased, and cells develop as adherent colonies with less invasiveness when ER α is forced to get expressed. ER α knockdown, on the other hand, causes an increase in Slug expression, a decrease in E-cadherin, and spindle-shaped invasive cells.

-In luminal-type breast tumours, E2 signalling modulates the activity of ER α and associated cofactors GATA3 and FOXA1 to promote an epithelial phenotype and repress EMT and pro-metastatic progression. In vitro, direct regulatory linkages have been shown, such as increased invasion and beginning of EMT in MCF-7 cells after overexpression of RELB or Snail due to suppression of ER α and E-cadherin, and in vivo results support these findings.

-In breast cancer patients, high levels of ER α , GATA3, and FOXA1 expression are linked to a better prognosis. Invasive basal-like and claudin-low breast cancer subtypes, on the other hand, are associated with high levels of RELB and Snail expression (as well as no/low expression of ER α , GATA3, and FOXA1).

• Wik et al used integrated molecular profiling to examine Endometrial cancer samples from a primary investigation cohort and three independent validation cohorts (Wik et al.,2013). Patient survival was closely linked to ER- α immunohistochemical staining and receptor gene (ESR1) mRNA expression. In the study cohort, ER- α negative was related with activation of genes implicated in Wnt, Sonic Hedgehog, and TGF- β signalling, indicating epithelial–mesenchymal transition (EMT).

-In conclusion, the absence of ER- α in endometrial cancer is linked to EMT and a shorter survival time.

• Bouris et al used specialised shRNA lentiviral particles to create stably transfected MCF-7 cells by knocking down the ER gene (identified as MCF-7/SP10 + cells) and compared them to control cells (MCF-7/c). In MCF-7 cells, ER suppression triggered cellular phenotypic changes as well as significant changes in gene and protein expression of many markers associated with epithelial to mesenchymal transition (EMT). These cells, in particular, showed increased cell proliferation, migration, and invasion(Bouris et al.,2015).

• N1-Guanyl-1,7-Diaminoheptane Sensitizes Estrogen Receptor Negative Breast Cancer Cells to Doxorubicin by Preventing Epithelial-Mesenchymal Transition by Inhibiting Eukaryotic Translation Initiation Factor 5A2 Activation (Liu et al.,2015)

• Saleh et al. hypothesise that loss of oestrogen receptor function, which causes endocrine resistance in breast cancer, also causes trans-differentiation from an epithelial to a mesenchymal phenotype, which causes enhanced aggressiveness and metastatic tendency(Saleh et al., 2011).

-siRNA-mediated oestrogen receptor silencing in MCF7 breast cancer cells resulted in estrogen/tamoxifen resistant cells (pII) with altered morphology, increased motility with cytoskeleton rearrangement and switch from keratin/actin to vimentin, and ability to invade simulated extracellular matrix components.

-Invasion assay of MCF7, E2, pII and MDA231 cells through simulated ECM protein components was carried out.Cells were plated into the upper chambers of the cell invasion plate and incubated for 48 h prior to measurement of the fluorescence intensity of the invading cells in the bottom chambers. MCF7 and E2 cells were considered non- invasive (<20 FU/m), whereas pII and MDA231 cells progressively invaded the BME (100 FU/m).

• Quantitative proteomics demonstrates ER participation in CD146-induced epithelial-mesenchymal transition in breast cancer cells, according to Zheng et al. They used a three-step Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) method to examine whole cell protein profiles of MCF-7 cells that had undergone EMT as CD146 expression increased from moderate to high levels (Zheng et al.,2014).

-In total, 2293 proteins were identified in this investigation, with 103 showing changes in protein abundance that linked with CD146 expression levels, demonstrating substantial morphological and biochemical changes associated with EMT.

-According to the Ingenuity Pathway Analysis (IPA), oestrogen receptor (ER) was the transcription regulator that was most strongly suppressed during CD146-induced EMT. In addition, functional experiments demonstrated that in cells undergoing CD146-induced EMT, ER expression was suppressed, but re-expression of ER eliminated their migratory and invasive characteristics. Finally, we discovered that ER- exerted its effects on CD146-induced EMT via inhibiting Slug, a major EMT transcriptional component.

• By immunohistochemistry, Ye et al found a high direct link between ER α and E-cadherin expression in human breast tumours, showing that ER α signalling may regulate E-cadherin and influence EMT and tumour growth(Ye et al.,2010).They looked at the impacts of ER α signalling in ER α -transfected ER α -negative breast carcinoma cell lines MDA-MB-468 and MDA-MB-231, as well as the effects of ER α knockdown in naturally expressing ER α -positive lines MCF-7 and T47D, to test this theory and the mechanisms behind it.17 β -estradiol (E2) decreased slug and increased E-cadherin in ER α -negative lines when ER α was overexpressed.

-In Matrigel, clones that showed the most of these alterations developed in clusters and were less invasive.Slug rose, E-cadherin reduced, cells became spindly, and Matrigel invasion increased when ER α was knocked down in ER α -positive lines. Slug expression was reduced by ER α signalling in two ways: directly, by repression of slug transcription via the formation of a corepressor complex containing ligand-activated ER α , HDAC inhibitor (HDAC1), and nuclear receptor corepressor (N-CoR) that bound the slug promoter in three half-site oestrogen response elements (EREs); and indirectly, by phosphorylation and inactivation of GSK-3 β via phosphoinositide (Akt). Inactivation of GSK-3 β suppressed slug expression while increasing E-cadherin. There was a substantial inverse connection between slug and ER α and E-cadherin immunoreactivity in human breast cancer cases. The data suggest that E-cadherin and EMT are regulated by ER α signalling through slug.

Uncertainties and Inconsistencies

No specific Uncertainties and Inconsistencies noted to the best of our knowledge.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human cell line	qRT-PCR, cell viability assay, Western blotting, EdU incorporation assay (C1,c2,C3,C5,c6,c8)	Yes	Strong	Yes	Yes	Yes
Human	IHC,microarray,qPCR, SNP array(H)	Yes	Moderate	Yes	Yes	Yes

Response-response relationship

- Endogenous ER silencing causes EMT in ER-positive breast cancer cells.

ER-positive MCF-7 cells were infected with ER shRNA lentiviral particles and stable clones were selected with puromycin (optimal dose of 0.8 g/mL) to knockdown ER gene expression (Zheng et al.,2014).

-When the number of cell passages was increased following infection, the expression of ER was gradually knocked down.

-ER gene expression was decreased by roughly 25% four passages after infection compared to control lentiviral particles transfected cells (MCF-7/c cells). The ER gene expression was lowered even more in the following passage (passage 5 post-infection) (by around 50% compared to MCF-7/c cells). In passage 7, a significant reduction in ER gene expression (about 75–80%) was seen, along with a distinct transition of cells from an epithelial to a mesenchymal phenotype.

- When MCF-7 cells reach confluency, they develop as closely packed colonies that produce sheet-like monolayer structures. Stable clones from stage 7 post-infection, on the other hand, grew as more elongated individual cells rather than tight clusters, with a spindle-like shape. For stable clones with a distinct mesenchymal character, a very substantial down-regulation of ER gene expression (above 99 percent) was found from passage 10 and beyond. MCF-7/SP10+ was given to these cells to emphasise the stable transfection (S) and passage 10 or more (P10+). The substantial down-regulation of ERα was confirmed by immunofluorescence and Western blot analysis of the same stable clones (MCF-7/ SP10 + cells).

Time-scale

Downstream key event occurs within hours of the occurrence of the upstream key event.

Known modulating factors

Tumour characteristics and heterogeneity, biological changes of tumour progression and interacting molecules, all of which can influence the degree of hormone responsiveness in a particular individual with hormone receptor-positive breast cancer.

Known Feedforward/Feedback loops influencing this KER

EMT is inhibited by ERα, and microRNAs either promote or inhibit EMT . These findings raise the question of whether microRNAs have a role in the control of EMT by targeting ERα mRNA. The large (>4000 nt) 30 untranslated region (30-UTR) of human ERα mRNA, as well as results that particular microRNAs are differentially expressed between ERα-positive and ERα-negative breast tumours , suggest the possibility of microRNA-mediated control of human ERα mRNA(Adams et al.,2008).

Pro-metastatic/anti-proliferative (miR-206), pro-metastatic/pro-proliferative (miR-221/222), and anti-proliferative/anti-metastatic (miR-221/223) ERα-targeting microRNAs (miR-130a, miR-145). miR-17/92 appears to be prometastatic, although it is implicated in several feedback loops, which could make miR-17/92's expression and effects on proliferation extremely reliant on the microenvironment as well as the genetic and epigenetic background.

Accurate identification of micro-RNAs that contribute significantly to a particular pathway (such as EMT) within breast cancers in situ is one hurdle. MicroRNAs have hundreds of potential targets, and in vivo studies will be needed to identify physiologically important targets in the context of breast cancer, as well as to develop effective treatments for breast cancer that involve manipulating microRNA expression levels and identifying off-target effects. (Adams et al.,2007;Zhao et al.,2008;Leva et al.,2010;Stinson et al.,2011;Acunzo et al.,2011;Castellano et al.,2009).

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Relationship: 2614: EMT leads to Metastasis, Breast Cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	High

EMT induces cancer invasion, metastasis (*Homo sapiens*)([P. Zhang et al., 2015](#)).

EMT is related to cancer drug resistance in MCF-7 human breast cancer cells (*Homo sapiens*)([B. Du & Shim, 2016](#)).

Key Event Relationship Description

Upstream event: Increased, EMT

Downstream event: Metastasis

Evidence Supporting this KER

The "epithelial-mesenchymal transition" (EMT), a key developmental regulatory program, has been reported to play critical and intricate roles in promoting tumor invasion and metastasis in epithelium-derived carcinomas in recent years. The EMT program allows stationary and polarized epithelial cells, which are connected laterally via several types of junctions and normally interact with the basement membrane via their basal surfaces to maintain apical-basal polarity, to undergo multiple biochemical changes that enable them to disrupt cell-cell adherence, lose apical-basal polarity, dramatically remodel the cytoskeleton, and acquire mesenchymal characteristics such as enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components. (Boyer et al., 1993). Some of the cells undergoing EMT have the characteristics of cancer stem cells (CSCs), which are linked to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015). Cancer metastasis and cancer therapeutic resistance are linked to the EMT phenomenon (Smith & Bhowmick, 2016; Tanabe, 2013). EMT causes the cell to escape from the basement membrane and metastasize by increasing the production of enzymes that breakdown extracellular matrix components and decreasing adherence to the basement membrane (Smith & Bhowmick, 2016). Therapy resistance is linked to morphological alterations seen during EMT (Smith & Bhowmick, 2016).

Biological Plausibility

Biological Plausibility

EMT is marked by a decrease in E-cadherin and β -catenin expression and an increase in vimentin, fibronectin, and N-cadherin expression (Irani et al., 2018). EMT is a master mechanism in cancer cells that allows them to lose their epithelial characteristics and gain mesenchymal-like qualities. EMT is the most crucial step in initiating metastasis, including metastasis to lymph nodes, because tumour cell movement is a pre-requisite for the metastatic process (Da et al., 2017). Multiple signalling pathways cause cancer cells to lose their cell-to-cell connections and cellular polarity during EMT, increasing their motility and invasive ness (Huang et al., 2017). MMPs cause E-cadherin to be cleaved, which increases tumour cell motility and invasion (Pradella et al., 2017).

Invasiveness and medication resistance are linked to the morphological and physiological changes associated with EMT (Shibue & Weinberg, 2017). In initial tumours, EMT-activated carcinoma cells penetrate the surrounding stroma (Shibue & Weinberg, 2017). EMT-activated carcinoma cells interact with the extracellular matrix protein to activate focal adhesion kinase and extracellular signal-related kinase, followed by TGF β and canonical and/or noncanonical Wnt pathways to develop cancer stem cell (CSC) traits, which contribute to drug resistance (Shibue & Weinberg, 2017).

Drug efflux and cell proliferation are slowed by EMT-associated downregulation of several apoptotic signalling pathways, resulting in general resistance of carcinoma cells to anti-cancer drugs (Shibue & Weinberg, 2017). Snail, an EMT-related transcription factor, promotes the production of the AXL receptor tyrosine kinase, which allows cancer cells to survive by activating AXL signalling when its ligand, growth arrest-specific protein 6 (GAS6), binds to it (Shibue & Weinberg, 2017).

EMT-activated cells are resistant to the deadly effects of cytotoxic T cells, which include increased expression of programmed cell death 1 ligand (PD-L1), which binds to the inhibitory immune-checkpoint receptor programmed cell death protein 1 (PD-1) on the cell surface of cytotoxic T cells (Shibue & Weinberg, 2017).

The reversing process of EMT, which names as a mesenchymal-epithelial transition (MET), maybe one of the candidates for the anti-cancer therapy, where the plasticity of the cell phenotype is of importance and under investigation (Shibue & Weinberg, 2017).

Empirical Evidence

Incidence concordance

- By inhibiting PUMA (also known as BBC3, encoding Bcl-2-binding component 3) and conferring resistance to p53-mediated apoptosis of hematopoietic progenitors, Slug/Snai2, a ces-1-related zinc finger transcription factor gene, confers resistance to p53-mediated apoptosis of hematopoietic progenitors (Inukai et al., 1999; Shibue & Weinberg, 2017; W.-S. Wu et al., 2005). TGF β -1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties by inducing the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in doxorubicin resistance (Saxena et al., 2011; Shibue & Weinberg, 2017). (Pirozzi et al., 2011; Shibue & Weinberg, 2017). Cancer metastasis and resistance to dendritic cell-mediated immunotherapy are promoted by snail-driven EMT (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009). EMT induced by the zinc finger E-box-binding homeobox (ZEB1) relieves miR-200-mediated repression of programmed cell death 1 ligand (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein (PD-1) immune-checkpoint protein on CD8⁺ cytotoxic T lymphocytes (CTL), resulting in immunosuppression and metastasis of CD8⁺ T cells (Chen et al., 2014).
- Wnt signalling is important for embryonic development, and genetic abnormalities in this network have been linked to colorectal cancer (Gujral et al., 2014). The Wnt receptor Frizzled2 (Fzd2) and its ligands Wnt5a/b are enhanced in metastatic liver, lung, colon, and breast cancer cell lines and in high-grade malignancies, and their expression correlates with epithelial-mesenchymal transition markers (EMT) (They created an anti-Fzd2 antibody that decreases tumour growth and metastasis in xenografts by reducing cell migration and invasion (Support for essentiality). Patients with malignancies that exhibit high levels of Fzd2 and Wnt5a/b may benefit from blocking this pathway, according to the researchers.
- In breast, colon, liver, and 186 lung cancer cell lines, researchers discovered a link between Fzd2 and its ligands Wnt5a/b and mesenchymal markers.
 - Fzd2 mRNA expression is significantly increased in late stages (stages III and IV) of primary liver and lung cancers compared with normal tissue
- Fzd2 regulated cell migration.
- Fzd2 signaling regulates EMT program
- expression of Fzd2 in Huh7 cells decreased levels of the epithelial markers E-cadherin and Occludin and increased levels of the mesenchymal markers Foxc1 and Vimentin.
 - Exposing cells to an inhibitor of Wnt secretion (C59) decreased Stat3 transcriptional activity in FOCUS cells 2- to 4-fold, whereas overexpressing Fzd2 in Huh7 cells increased Stat3 activity 2-fold.
- Cui et al discovered a link between metastasis and the expression of ROR1, a type I receptor tyrosine kinase-like orphan receptor that is expressed throughout embryogenesis and by a variety of malignancies but not by normal postpartum tissues (Cui et al., 2013). ROR1 expression has been linked to the epithelial-mesenchymal transition (EMT), which occurs during embryogenesis and cancer metastasis, according to their findings. Breast adenocarcinomas with high ROR1 expression were more likely to have gene expression profiles consistent with EMT and had greater rates of recurrence and metastasis than those with low ROR1 expression. Suppressing ROR1 expression in metastasis-prone breast cancer cell lines MDA-MB-231, HS-578T, or BT549 decreased expression of proteins associated with EMT (e.g., vimentin, SNAIL-1/2, and ZEB1), increased expression of E-cadherin, epithelial cytokeratins (e.g., CK-19), and tight junction proteins (e.g., ZO-1), and impaired their migration/invasion capacity in vitro. (Support for essentiality)
- Conversely, transfection of MCF-7 cells to express ROR1 reduced expression of E-cadherin and CK-19, but enhanced the expression of SNAIL-1/2 and vimentin. Treatment of MDA-MB-231 with a monoclonal antibody specific for ROR1 induced downmodulation of vimentin and inhibited cancer cell migration and invasion in vitro and tumor metastasis in vivo.
 - ROR1 associates with metastatic cancer phenotypes
 - ROR1 is associated with early metastatic relapse in breast adenocarcinoma
 - Silencing ROR1 inhibits orthotopic lung metastasis
 - Silencing ROR1 inhibits experimental lung and bone metastasis
 - An anti-ROR1 antibody inhibits cancer metastasis (Support for essentiality)
- At 37°C, monoclonal antibodies (mAb) specific for ROR1's extracellular domain were created, and one (D10) was chosen to elicit fast downmodulation of surface ROR1. ROR1 internalisation was observed in MDA-MB-231 cells treated with D10, as determined by confocal microscopy. As measured by flow cytometry with a separate mAb specific for a unique, non-cross-blocking epitope of ROR1, this resulted in a considerable reduction in ROR1. D10 treatment of MDA-MB-231 reduced cytoplasmic vimentin expression, which was bound to ROR1 in coimmunoprecipitation studies. In vitro, D10 treatment significantly reduced the ability of MDA-MB-231 to migrate and invade. D10 may also be able to stop other ROR1 cancer cell lines from migrating or invading.
- Chen et al investigated the potential function of MDM2 in ovarian cancer SKOV3 cells' EMT and metastasis (Chen et al., 2015).
- Wound-healing and transwell tests were used to mimic MDM2's regulatory effects on cell motility. By displaying the expression levels of epithelial marker E-cadherin as well as critical components of the Smad pathway, the impacts on EMT transition and Smad pathway were explored. The connection of MDM2 expression levels with the stages of 104 ovarian cancer patients was explored using an immunohistochemical assay to assess the clinical relevance of their findings.
- Results show that MDM2 plays a significant role in driving EMT and motility in ovarian SKOV3 cells by promoting the activation of the TGF- β -Smad pathway, which leads to increased snail/slug transcription and a decrease of E-cadherin levels.
- Such induction of EMT is sustained in either E3 ligase-depleted MDM2 or E3 ligase inhibitor HU-373-treated cells, but is reduced by MDM2 N-terminal deletion, as evidenced by Nutlin-3a, the N-terminal targeting agent's inhibitory effects on EMT. MDM2 expression levels are substantially correlated with ovarian cancer stages, and increased MDM2 expression in combination with TGFB is associated with a bad prognosis and predicts a high risk of ovarian cancer patients.
- This research suggests that MDM2 activates the Smad pathway to promote EMT in ovarian cancer metastasis, and that targeting MDM2's N-terminal can reprogram EMT and limit cancer cell mobility.
- HOXD9, a Hox family member, is involved in cancer growth and metastasis. But, its regulation mechanism at the molecular level particularly in colo rectal cancer (CRC), is mostly unknown. Liu and colleagues used immunofluorescence, immunohistochemistry (IHC), and western blot to examine the levels of HOXD9 protein expression. Colony formation and EdU incorporation, CCK-8, wound scratch and transwell invasion assays, and animal models were used to determine the in vivo and in vitro roles of HOXD9 in CRC. In CRC, HOXD9 expression was higher than in matched healthy tissues (Liu et al., 2020). High HOXD9 expression has been linked to advanced stages of cancer, tumour differentiation, lymph node metastasis, and other serious invasions, as well as a poor prognosis, according to the American Joint Committee on Cancer (AJCC). In CRC cells, HOXD9 promoted proliferation, motility, and EMT processes in vitro. TGF-1 also stimulated the expression of HOXD9, which was dosage dependent, and HOXD9 downregulation suppressed TGF-1-induced EMT. Through orthotopic implantation, HOXD9 promoted the invasiveness and metastasis of CRC cells in vivo. The ectopic expression of HOXD9 promoted the invasion metastasis in cells of the colorectal tumor by induction of EMT in vitro and vivo.
- Twist1, Snail1, Snail2, ZEB1, and ZEB2 are among a group of transcription factors that have been demonstrated to promote tumour spread by inducing epithelial mesenchymal transition (EMT). However, it is unknown whether these transcription factors activate the EMT program separately or in concert. Twist1 requires direct induction of Snail2 to induce EMT, according to the study by Casas et al. Twist1's capacity to decrease E-cadherin transcription is totally blocked when Snail2 is knocked off. Twist1 induces Snail2 transcription by binding to an evolutionarily conserved E-box on the proximal promoter. Twist1-induced cell invasion and distant metastasis in mice require Snail2 induction. Twist1 and Snail2 expression in human breast cancers are significantly linked. Results of the study by Casas et al show that Twist1 needs to induce Snail2 to suppress the epithelial branch of the EMT program and that Twist1 and Snail2 act together to promote EMT and tumor metastasis in human mammary epithelial cell lines (Casas et al., 2012).
- The basic helix-loop-helix transcription factor AP4/TFAP4/AP-4 is encoded by a c-MYC target gene and is up-regulated in colorectal cancer (CRC) and a variety of other tumour types at the same time as c-MYC. A combination of microarray, genome-wide chromatin immunoprecipitation, next-generation sequencing, and bioinformatic studies were used to characterise AP4 DNA binding and mRNA expression across the genome. Hundreds of AP4 target genes were identified as activated and repressed as a result. SNAIL, E-cadherin/CDH1, OCLN, VIM, FN1, and the Claudins 1, 4, and 7 were among the AP4 target genes, which included markers of stemness (LGR5 and CD44) and epithelial-mesenchymal transition (EMT) such as SNAIL, E-cadherin/CDH1, OCLN, VIM, and FN1. As a result, AP4 activation promoted EMT and increased CRC cell motility and invasion. Down-

- regulation of AP4 hindered migration and invasion by causing mesenchymal–epithelial transition (Jackstadt et al., 2013).
- EMT, migration, and invasion produced by ectopic expression of c-MYC also needed AP4 induction. Lung metastasis in mice was reduced when AP4 was inhibited in CRC cells. Increased AP4 expression was linked to liver metastases and poor patient survival in primary CRC. These findings point to AP4 as a novel EMT regulator that plays a role in CRC and maybe other carcinomas’ metastatic processes.

Uncertainties and Inconsistencies

Whenever cell phenotype plasticity is crucial and under investigation, the reverse of EMT, known as the mesenchymal-epithelial transition (MET), may be one of the prospects for anti-cancer therapy (Shibue & Weinberg, 2017).

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Cell line,humans,Human cell line studies	qRT-PCR,,Luciferase reporter assay ,immunoblotting,immunoprecipitation,cell invasion assay,cell migration assay, bioluminescence imaging,wound healing assay,Wound scratch & Transwell assay, Microarray,Immunofluorescence, Immunohistochemistry (table 1)	Yes	Strong	Yes	Yes	Yes

Response-response relationship

TGFbeta and Twist induce EMT by upregulating the expression of EMT markers such Snail, Vimentin, N-cadherin, and ABC transporters like ABCA3, ABCC1, ABCC3, and ABCC10 (Saxena et al., 2011).In the treatment with about 0.3, 3, 30 mM of doxorubicin, human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrate increased cell viability compared to control HMLE, dose-dependently (Saxena et al., 2011).

Time-scale

When Twist/FOXC2/Snail overexpressed HMLE is treated with doxorubicin for 48 hours, cell viability increases compared to control HMLE (Saxena et al., 2011).When Twist or Zeb1 were inhibited with small interference RNA (siRNA), cell viability was reduced relative to control MDAMB231 cells treated with doxorubicin for 48 hours (Saxena et al., 2011).

Known modulating factors

In EMT-activated cells, ABC transporters linked to drug resistance are overexpressed (Saxena et al., 2011). In EMT-activated cells, the expression of PD-L1, which binds to PD-1 on cytotoxic T cells, is upregulated, inhibiting cancer immunity and increasing resistance to cancer therapy (Shibue & Weinberg, 2017).

Known Feedforward/Feedback loops influencing this KER

- Understanding the association between EMT and cancer malignancy necessitates further research into the EMT-cancer stem cells (CSC) relationship. Non-CSCs in cancer can spontaneously undergo EMT and dedifferentiate into new CSCs, resulting in tumorigenic potential renewal (Marjanovic, Weinberg, & Chaffer, 2013; Shibue & Weinberg, 2017).The plastic CSC theory demonstrates bidirectional conversions between non-CSCs and CSCs, which could help EMT-activated cells acquire cancer malignancy (Marjanovic et al., 2013).
- Long non-coding RNAs (lncRNAs) play crucial roles in many biological and pathological processes, including tumor metastasis. Kong et al reported a novel lncRNA, LINC01133 that was downregulated by TGF- β, which could inhibit epithelial–mesenchymal transition (EMT) and metastasis in colorectal cancer (CRC) cells (Kong et al.,2016). SRSF6, an alternative splicing factor that interacts directly with LINC01133, was found to enhance EMT and metastasis in CRC cells even when LINC01133 was not present. The study also found that the EMT process in CRC cells was regulated by LINC01133 in the presence of SRSF6. In vivo, the ability of LINC01133 to prevent metastasis was confirmed. Furthermore, clinical data revealed that LINC01133 expression was favourably correlated with E-cadherin and negatively correlated with Vimentin, and that low LINC01133 expression in tumours was associated with poor CRC survival. These findings show that LINC01133, by directly binding to SRSF6 as a target mimic and inhibiting EMT and metastasis, could be used as a predictive biomarker and an effective target for anti-metastasis therapy in CRC.
- MiR-148a inhibited Met expression directly by binding to its 30-UTR, according to Zhang et al's findings. Furthermore, reintroducing miR-148a reduced the nuclear accumulation of Snail, a transcription factor that promotes EMT, by inhibiting Met's downstream signalling, such as activating phosphorylation of AKT-Ser473 and inhibitory phosphorylation of GSK-3b-Ser9 (Zhang et al.,2015). MiR-148a, when combined, may suppress hepatoma cell EMT and metastasis by adversely regulating Met/Snail signalling.

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List of Non Adjacent Key Event Relationships

[Relationship: 2728: Increased, DNA damage and mutation leads to Increase chromosomal aberrations](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	High	NCBI
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Mixed	Not Specified

Not specific,

Key Event Relationship Description

Increased DNA damage leads to increased chromosomal aberrations

Evidence Supporting this KER

DNA double-strand breaks (DSB) are the crucial lesions underlying the formation of CA [M.A Bender et al.,1974; G.Obe et al., 2002]. Chromosomes are uninemic; each chromatid contains one continuous DNA molecule. Consequently, an unrepaired DSB, appears at mitosis as a terminal deletion (or an incomplete exchange), leading to loss of genetic material and eventually cell death or loss of heterozygosity in diploid cells. On the other hand, misrepaired DSB generate intra- or inter-chromosomal exchanges which may or may not be lethal, depending on the exact form they take. Concluding a controversy that lasted a number of years ([K.H Chandwick et al.,1981] and references therein), there is now a general agreement that the dose–response curve for the induction of DSB is linear over several orders of magnitude [K Rothkamm et al., 2003]

Biological Plausibility

DNA damage and unrepaired or insufficiently repaired DNA double-strand breaks as well as telomere shortening contribute to the formation of structural chromosomal aberrations (CAs). Non-specific CAs have been used in the monitoring of individuals exposed to potential carcinogenic chemicals and radiation. The frequency of CAs in peripheral blood lymphocytes (PBLs) has been associated with cancer risk and the association has also been found in incident cancer patients. CAs include chromosome type aberrations (CSAs) and chromatid-type aberrations (CTAs) and their sum CA_{tot}.

Structural CAs may be specific, such as translocations and inversions, or non-specific, such as chromatid breaks, fragmented or missing parts of chromosomes, and fusions resulting in dicentric and ring chromosomes (Bignold, 2009).

The former are often recurrent and they are currently analyzed by molecular cytogenetic methods while the latter are scored by classical cytogenetic techniques, which are able to recognize chromosome-type aberrations (CSAs) and chromatid-type aberrations (CTAs) according to morphological changes (Hagmar et al., 2004). CTAs are formed due to insufficiently repaired double-strand breaks (DS₀₀Bs) during the late S or G2 phase of the

cell cycle (Natarajan and Palitti, 2008; Bignold, 2009; Durante et al., 2013), whereas CSAs are the result of direct DNA damage due to radiation, chemical mutagens, or shortening of telomeres during the G0/G1 phase (Albertini et al., 20; Jones et al., 2012). Non-specific CAs have been used in the monitoring of populations occupationally exposed to potential carcinogenic chemicals and radiation and an increased frequency of CAs in peripheral blood lymphocytes (PBLs) has been associated with cancer risk and the association has also been found in incident cancer patients (Rossner et al., 2005; Vodicka et al., 2010; Vodenkova et al., 2015).

Unrepaired or insufficiently repaired DSBs, as well as telomerase dysfunction, represent the mechanistic bases for the formation of structural CAs (Natarajan and Palitti, 2008;

Bignold, 2009; Durante et al., 2013; Vodicka et al., 2018; Srinivas et al., 2020). However, even other types of DNA repair pathways may contribute to CA formation as these are found in inherited syndromes manifesting DNA repair gene mutations (Rahman, 2014).

Empirical Evidence

A study by Solange et al evaluated the effects of exposure to formaldehyde (FA) in human peripheral blood lymphocytes, a group of laboratory workers exposed occupationally to FA and control subjects were tested for chromosomal aberrations (CAs) and DNA damage (comet assay) (Solange et al 2015). The level of exposure to FA in the workplace air was evaluated. The association between genotoxicity biomarkers and polymorphic genes of xenobiotic - metabolising and DNA repair enzymes were also assessed.

All cytogenetic parameters evaluated—total-CA, CSAs, CTAs, gaps and aneuploidies—were significantly elevated in anatomy pathology professionals exposed to FA (mean 0.38 ppm) compared with control subjects. FA-exposed individuals showed an increase of 91% in total-CAs frequency compared with controls. Mean frequencies of both CAs types, CSAs and CTAs were also significantly higher in exposed workers. Although there is a paucity of studies assessing CAs in FA occupationally exposed subjects, our findings are in agreement with most of the published literature. (He.J.L. *et al.* 1998) found higher frequencies of CAs in PBLs of 13 anatomy students exposed to FA (mean level 2.37 ppm) during a 12-week anatomy class. Similarly, in a recent study involving FA-exposed personnel working in pathology departments ($n = 21$; mean level 0.72 ppm), total-CA and CTAs were significantly elevated

compared with controls (Jakab et al., 2010). A significant increase in CAs frequencies was also observed in industrial workers (Kitaeva et al., 1996).

Multiaberrant cells frequency was significantly higher (4-fold) in FA-exposed workers than in control individuals, whereas aberrant cells frequency was significantly increased by 1.7-fold in the exposed group (Solange et al., 2015).

Uncertainties and Inconsistencies

In contrast, no significant differences were found in CAs frequencies between individuals working in different laboratories of a Cancer Research Institute, including an anatomical pathology laboratory (Pala M et al., 2008)

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Mice	Chromosomal aberration assay, Genotoxicity assessment assay, (Evgenii Plotnikov., et al 2016) CT8 Assay (Francesco Marchetti., et al 2015)	+	Strong	Yes	Yes	Yes
Human	Micronuclease (CBMN) Assay, Comet assay, (Qiang Liu., et al 2009.) CAs analysis, Comet assay, PCR-RFLP (S. Costa et al., 2015)	+	Strong	Yes	Yes	Yes
Human Cell lines	Polyploid assay, Sister chromatid exchange test, V79/HPRT mutation assay, Cell transformation assay, Tumorigenicity test, spore rec assay (Hirohisa Tsuda et al., 1993)	+	Strong	Yes	Yes	Yes

Response-response relationship

Multiaberrant cells frequency was significantly higher (4-fold) in formaldehyde-exposed workers than in control individuals, whereas aberrant cells frequency was significantly increased by 1.7-fold in the exposed group (Solange et al., 2015).

Time-scale

It is generally accepted that exchanges formed in the G1-phase originate from the interaction of two spatially distinct radiogenic damaged sites (DSB) [Heck et al., 2008], which runs counter to the once-popular concept encompassed by so-called "one-hit models" for the formation of translocations, dicentrics and other exchanges [Pala M et al., 2008].

Known Feedforward/Feedback loops influencing this KER

Not known.

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Relationship: 2729: Increase chromosomal aberrations leads to Increase miRNA levels

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
DNA damage and mutations leading to Metastatic Breast Cancer	non-adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Mixed	High

Not specific through any particular life stage or gender

Key Event Relationship Description

KER :increased, chromosomal aberration leads to microRNA expression, increased

Upstream event: increased, chromosomal aberration

Downstream event: increased miRNA expression

Evidence Supporting this KER

The first report linking a chromosomal breakpoint with the genomic location of miRNAs was published a couple of decades ago (Gauwerky et al.,1989). A masked t(8;17) translocation resulted in a high activation of the MYC OG: MYC from chromosome 8 was truncated at the end of the first exon (which is noncoding), and the coding region joined the regulatory elements of a gene located on chromosome 17, called BCL3 (B cell leukemia/lymphoma 3). Despite extensive genomic search, BCL3 remained an elusive entity until the identification of the human miRNAs. Fifteen years after the initial discovery, the miR-142 gene was found to be located 50 nt from the t(8;17) break involving chromosome 17 and MYC, meaning that the regulatory elements of this miRNA are likely involved in the overexpression of MYC (Calin, G.A., et al. 2002). The clinical consequences were dramatic for the patient, leading to aggressive acute prolymphocytic leukemia (Gauwerky et al.,1989). Apart from the involvement in the t(8;17) breakpoint of B cell acute leukemia, miR-142-3p and miR-142-5p are also within the 17q23 minimal amplicon described in breast cancer (Barlund, M., et al. 2000) and near the FRA17B site, a target for HPV16 integration in cervical tumors (Calin, G.A., et al. 2004).

Biological Plausibility

Chromosomal translocations alter PCG loci through two main mechanisms (Russo, G., et al. 1988). The first is the juxtaposition of promoter/enhancer elements from one gene to the intact coding region of another gene, while the second is the recombination of the coding regions of two different genes. The former is more frequently found in B and T cell lymphomas and leukemias and the latter in human myeloid leukemias and soft-tissue sarcomas. The translocations that alter miRNA loci can be classified by analogy with these mechanisms. At least five different situations can be postulated, the last three of which have yet to be identified in human cancers: (a) juxtaposition of promoter/enhancer elements from miRNA genes to a PCG ORF with overexpression of the protein [e.g., t(8;17)(q24;q22)]; (b) disruption of the region of interaction between the target PCG and the interactor miRNA with the disruption of the repression and the overexpression of the protein (e.g., 12q15 translocations involving HMGA2 gene); (c) juxtaposition of promoter/enhancer elements from PCG to a miRNA gene with overexpression of the noncoding gene; (d) juxtaposition of promoter/enhancer elements from miRNA to another miRNA gene with overexpression of the noncoding gene (termed “promoter swapping”); and (e) miRNA gene-to-miRNA gene fusion with the consequent production of a “new” cluster of coexpressed or independently expressed miRNAs.

Empirical Evidence

miRNA genes are located near breakpoint regions. *miR-180* is only 1 kb from the *MN1* gene involved in a t(4;22) chromosomal translocation in meningioma that inactivates *MN1* and possibly the miRNA gene located in the same position. Also, in a patient with precursor B cell acute lymphoblastic leukemia, an insertion of *miR-125b-1* into a rearranged immunoglobulin heavy-chain locus was described, possibly as an early step in leukemogenesis (Sonoki, T et al., 2009-2010). Chromosomal translocations fusing the BCL-6 OG to the regulatory elements of *miR-28* or to the lipoma preferred partner were described in primary central nervous system lymphomas and may be associated with aberrant somatic hypermutation or defective class switch recombination (Schwindt, H., et al. 2006).

Uncertainties and Inconsistencies

The contribution of microRNAs (miR) to the pathogenesis of mantle cell lymphoma (MCL) is not well known. The expression of 86 mature miRs mapped to frequently altered genomic regions in MCL in CD5+ /CD5 normal B cells, reactive lymph nodes, and purified tumor cells of 17 leukemic MCL, 12 nodal MCL, and 8MCL cell lines were investigated. Genomic alterations of the tumors were studied by single nucleotide polymorphism arrays and comparative genomic hybridization. Leukemic and nodal tumors showed a high number of differentially expressed miRs compared with purified normal B cells, but only some of them were commonly deregulated in both tumor types. An unsupervised analysis of miR expression profile in purified leukemic MCL cells revealed two clusters of tumors characterized by different mutational status of the immunoglobulin genes, proliferation signature, and number of genomic alterations. The expression of most miRs was not related to copy number changes in their respective chromosomal loci. Only the levels of miRs included in the miR-17-92 cluster were significantly related to genetic alterations at 13q31. Moreover, overexpression of miR-17-5p/miR-20a from this cluster was associated with high MYC mRNA levels in tumors with a more aggressive behavior. In conclusion, the miR expression pattern of MCL is deregulated in comparison with normal lymphoid cells and distinguishes two subgroups of tumors with different biological features.

Quantitative Understanding of the Linkage

	Method/ measurement reference	Reliability	Strength of evidence	Assay fit for purpose	Repeatability/ reproducibility	Direct measure
Human	Microarray, analysis (dehan et al., 2007)	CGH+	Strong	Yes	Yes	Yes
Human cell line and blood samples	Northern blotting (Calin et al., 2004)	+	Strong	Yes	Yes	Yes
	Cytogenetic techniques (Lionetti M et al., 2009 ; Min DJ et al., 2013 ;Huang JJ et al., 2012 ; Pichiorri F, et al., 2011; Roccaro AM et al., 2009; Gao X et al., 2009 ; Corthals SL et al., 2010 ; Pichiorri F et al., 2008 ; Yang RF et al., 2010; Rio-Machin A et al., 2013 ; Gutiérrez NC et al., 2010 ; Kuehl WM et al., 2012 ; Pichiorri F et al., 2010 ; Gatt ME et al., 2011 ; Zhang Y-K et al., 2011 ;Misiewicz-Krzeminska J et al., 2013;Wong KY et al., 2011 ;Chim CS et al., 2010)	+	Strong	Yes	Yes	Yes

Response-response relationship

Detailed investigation of the 13q14.3 deletions showed that both members of an miRNA cluster, *miR-15a* and *miR-16-1*, are deleted or downregulated in approximately 68% of CLL cases as compared with healthy donors (Calin, G.A., et al. 2002). Furthermore, a rare mutation lowering the expression of these genes was identified in two CLL patients including one from a family with individuals having CLL and breast cancer, and was found to be associated with the loss of the normal allele in the leukemic cells (Calin, G.A., et al. 2005). It was shown that the levels of both *miR-15* and *miR-16* inversely correlate with the BCL-2 protein expression and that BCL-2 repression by these miRNAs induces apoptosis in leukemia cells (Cimmino, A., et al. 2005).

Levels of *miR-16* were decreased in NZB lymphoid tissue, and exogenous *miR-16* delivered to an NZB malignant B-1 cell line resulted in cell cycle alterations and increased apoptosis. Linkage of the *miR-15a/miR-16-1* complex to the development of CLL in this spontaneous mouse model suggests that the altered expression of these genes is the molecular lesion in CLL (Raveche, E.S., et al. 2007).

The only miRNA found to be overexpressed in any type of solid tumor analyzed (breast, colon, lung, prostate, stomach, and endocrine pancreas tumors, glioblastomas, and uterine leiomyomas) is *miR-21* (Volinia, S., et al. 2006, Ciafre, S.A., et al. 2005; Krichevsky et al., 2003; Wang, T., et al. 2007). This gene is located in the 3'UTR of the vacuole membrane protein 1 (*VMP1*) gene at chromosome 17q23.2, a region frequently found amplified in neuroblastomas and breast, colon, and lung cancers. Knockdown of *miR-21* in glioblastoma cell lines induces a caspase-mediated apoptosis, further supporting the oncogenic role of this miRNA (Chan, et al., 2005).

Time-scale

Studies performed in solid cancer cell lines showed that *miR-16* negatively regulated cellular growth and cell cycle progression. *miR-16*–downregulated transcripts were enriched with genes whose silencing by small interfering RNAs causes an accumulation of cells in G0/G1. Simultaneous silencing of these genes was more effective at blocking cell cycle progression than was disruption of the individual genes. Thus, *miR-16* coordinately regulates targets that may act in concert to control cell cycle progression (Linsley, P.S., et al. 2007)

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
UV rays, cisplatin, doxorubicin, IR	Impaired DNA repair	Altered miRNA expression	Pothof et al., 2009; Galluzzi et al., 2010, Saleh et al., 2011; Suzuki et al., 2009

Known Feedforward/Feedback loops influencing this KER

Not mentioned.

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