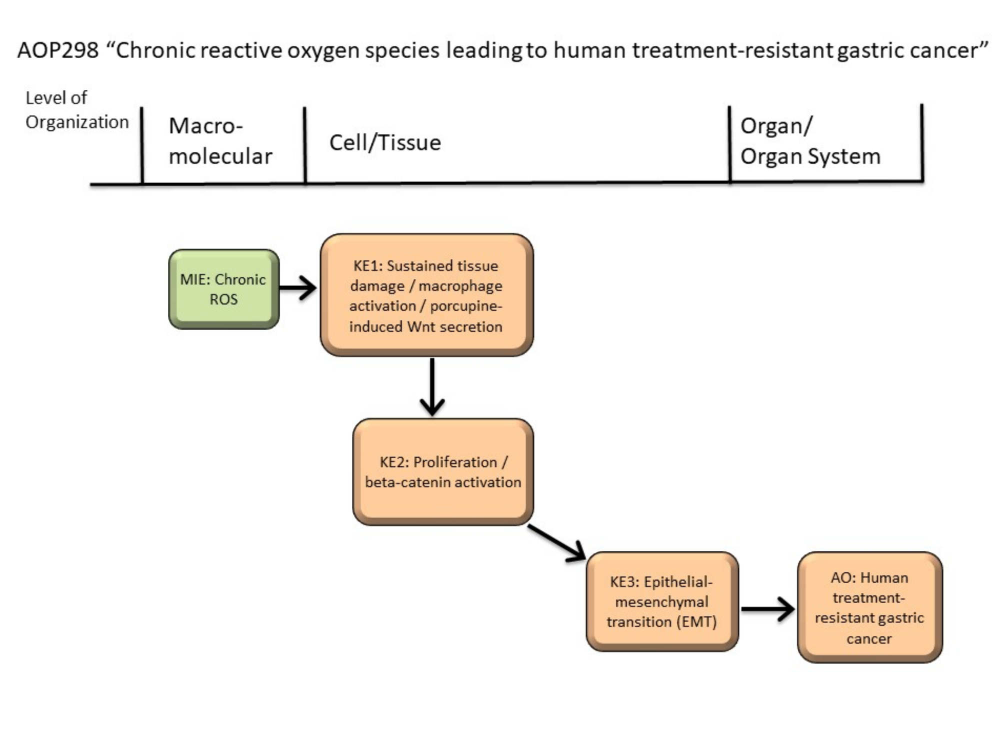
AOP ID and Title:

AOP 298: Chronic reactive oxygen species leading to human treatment-resistant gastric cancer
Short Title: Chronic ROS leading to human treatment-resistant gastric cancer

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for comment. Do not cite	EAGMST Under Review	1.58	Included in OECD Work Plan

Abstract

The injury or sustained reactive oxygen species (ROS) causes resistance in human gastric cancer. This AOP entitled "Chronic reactive oxygen species leading to human treatment-resistant gastric cancer" consists of MIE (KE1753) as chronic ROS, followed

by KE1 (KE1754) as sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion, KE2 (KE1755) as proliferation / beta-catenin activation, KE3 (KE1650) as epithelial-mesenchymal transition (EMT), and AO (KE1651) as human treatment-resistant gastric cancer. ROS has multiple roles such as development and progression of cancer, or apoptotic induction causing anti-tumor effects. In this AOP, we focus on the role of chronic ROS with sustained level to induce the therapy-resistance in human gastric cancer. EMT, which is cellular phenotypic change from epithelial to mesenchymal-like feature, demonstrates cancer stem cell-like characteristics in human gastric cancer. EMT is induced by Wnt/beta-catenin signaling, which confers rationale to have Wnt secretion and beta-catenin activation as KE1 and KE2 on the AOP, respectively.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1753	Chronic reactive oxygen species	Chronic ROS
2	KE	1940	Increases in cellular reactive oxygen species	Increases in cellular ROS
3	KE	1754	Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	Sustained tissue damage, macrophage activation and Wnt secretion
4	KE	1755	Proliferation / beta-catenin activation	Proliferation / beta-catenin activation
5	KE	1650	Epithelial-mesenchymal transition	Epithelial-mesenchymal transition
6	AO	1651	Treatment-resistant gastric cancer	Resistant gastric cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Chronic reactive oxygen species	adjacent	Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	Moderate	Moderate
Increases in cellular reactive oxygen species	adjacent	Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	Moderate	Moderate
Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	adjacent	Proliferation / beta-catenin activation	Moderate	Moderate
Proliferation / beta-catenin activation	adjacent	Epithelial-mesenchymal transition	Moderate	Moderate
Epithelial-mesenchymal transition	adjacent	Treatment-resistant gastric cancer	Moderate	Moderate

Stressors

Name	Evidence
Wnt	High
WNT2	High
Porcupine	Moderate
Wntless	Moderate
Ionizing Radiation	Moderate
ferric nitrilotriacetate	Not Specified

Wnt

WNT induces EMT (J. Zhang, Tian, & Xing, 2016).

WNT2

WNT2 induces EMT in cervical cancer (Zhou et al., 2016).

Porcupine

Porcupine palmitoleates Wnt and facilitates the secretion of the Wnt ligand (Yu & Virshup, 2014) .

Wntless

Wntless binds to and transport Wnt to the plasma membrane leading to the secretion of Wnt ligand (Yu & Virshup, 2014).

ferric nitrilotriacetate

Carcinogenic iron(III)-nitrilotriacetate induces reactive oxygen species production via trasfer of an electron to molecular oxygen to form reactive oxygen species [Tsuchiya K, Akai K, Tokumura A, Abe S, Tamaki T, Takiguchi Y, Fukuzawa K. Biochim Biophys Acta. 2005 Aug 30;1725(1):111-9. doi: 10.1016/j.bbagen.2005.05.001, Akai K, Tsuchiya K, Tokumura A, Kogure K, Ueno S, Shibata A, Tamaki T, Fukuzawa K. Free Radic Res. 2004 Sep;38(9):951-62. doi: 10.1080/1071576042000261945.].

Overall Assessment of the AOP

<table>
<tr><td colspan="2">1. Support for Biological Plausibility of KERs</td></tr>
<tr><td rowspan="2">MIE => KE1: Chronic ROS leads to Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion</td><td>Biological Plausibility of the MIE => KE1 is moderate.</td></tr>
<tr><td>Rationale: Sustained ROS increases caused by/causes DNA damage, which will alter several signaling pathways including Wnt signaling. Macrophages accumulate into injured tissue to recover the tissue damage, which may be followed by porcupine-induced Wnt secretion. ROS stimulate inflammatory factor production and Wnt/beta-catenin signaling (Vallée & Lecarpentier, 2018).</td></tr>
<tr><td rowspan="2">KE1 => KE2: Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion leads to Proliferation / beta-catenin activation</td><td>Biological Plausibility of the KE1 => KE2 is moderate.</td></tr>
<tr><td>Rationale: Secreted Wnt ligand stimulates Wnt/beta-catenin signaling, in which beta-catenin is activated. Wnt ligand binds to Frizzled receptor, which leads to GSK3beta inactivation. GSK3beta inactivation leads to beta-catenin dephosphorylation, which avoids the ubiquitination of the beta-catenin and stabilize the beta-catenin (Clevers & Nusse, 2012).</td></tr>
<tr><td rowspan="3">KE2 => KE3: Proliferation / beta-catenin activation leads to Epithelial-mesenchymal transition (EMT)</td><td>Biological Plausibility of the KE2 => KE3 is moderate.</td></tr>
<tr><td>Rationale: Beta-catenin activation, of which mechanism include the stabilization of the dephosphorylated beta-catenin and translocation of beta-catenin into the nucleus, induces the formation of beta-catenin-TCF complex and transcription of transcription factors such as Snail, Zeb and Twist (Clevers & Nusse, 2012) (Ahmad et al., 2012; Pearlman, Montes de Oca, Pal, & Afaq, 2017; Sohn et al., 2019; W. Yang et al., 2019).</td></tr>
<tr><td>EMT-related transcription factors including Snail, ZEB and Twist are up-regulated in cancer cells (Diaz, Vinas-Castells, & Garcia de Herreros, 2014). The transcription factors such as Snail, ZEB and Twist bind to E-cadherin (CDH1) promoter and inhibit the CDH1 transcription via the</td></tr>
</table>

	consensus E-boxes (5’-CACCTG-3’ or 5’-CAGGTG-3’), which leads to EMT (Diaz et al., 2014).
KE3 => AO: Epithelial-mesenchymal transition (EMT) leads to human treatment-resistant gastric cancer	Biological Plausibility of the KE3 => AO is moderate.
	Rationale: Some population of the cells exhibiting EMT demonstrates the feature of cancer stem cells (CSCs), which are related to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015).
	EMT phenomenon is related to cancer metastasis and cancer therapy resistance (Smith & Bhowmick, 2016; Tanabe, 2013). The increase in expression of enzymes that degrade the extracellular matrix components and the decrease in adhesion to the basement membrane in EMT induce the cell escape from the basement membrane and metastasis (Smith & Bhowmick, 2016). Morphological changes observed during EMT are associated with therapy resistance (Smith & Bhowmick, 2016).
2. Support for essentiality of KEs	
KE1: Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	Essentiality of the KE1 is moderate.
	Rationale for Essentiality of KEs in the AOP: The sustained tissue damage, macrophage activation and Wnt are essential for the subsequent beta-catenin activation and cancer resistance.
KE2: Proliferation / beta-catenin activation	Essentiality of the KE2 is moderate.
	Rationale for Essentiality of KEs in the AOP: Proliferation and beta-catenin activation are essential for the Wnt-induced cancer resistance.
KE3: Epithelial-mesenchymal transition (EMT)	Essentiality of the KE3 is moderate.
	Rationale for Essentiality of KEs in the AOP: EMT is essential for the Wnt-induced cancer promotion and resistance to anti-cancer drug.
3. Empirical support for KERs	
MIE => KE1: Chronic ROS leads to Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion	Empirical Support of the MIE => KE1 is moderate.
	Rationale: Production of ROS by DNA double-strand break causes the tissue damages (Gao et al., 2019).
	ROS signaling induces Wnt/beta-catenin signaling (Pérez et al., 2017).
KE1 => KE2: Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion leads to Proliferation / beta-catenin activation	Empirical Support of the KE1 => KE2 is moderate.
	Rationale: Sustained ROS increases caused by/causes DNA damage, which will alter several signaling pathways including Wnt signaling. Macrophages accumulate into injured tissue to recover the tissue damage, which may be followed by porcupine-induced Wnt secretion, which then activates beta-catenin leading to proliferation.
	Wnt binds to FZD and activate the Wnt signaling (Clevers & Nusse, 2012; Janda, Waghray, Levin, Thomas, & Garcia, 2012; Nile et al., 2017). Wnt binding towards FZD induce the formation of the protein complex with LRP5/6 and DVL, leading to the down-stream signaling activation including beta-catenin (Clevers & Nusse, 2012).
	Empirical Support of the KE2 => KE3 is moderate.

KE2 => KE3: Proliferation / beta-catenin activation leads to Epithelial-mesenchymal transition (EMT)	**Rationale: Proliferation and beta-catenin activation induces the key transcription factors in EMT. Translocation of beta-catenin into the nucleus by Wnt/beta-catenin signaling pathway activation promotes EMT.**
	The inhibition of c-MET, which is overexpressed in diffuse-type gastric cancer, induced increase in phosphorylated beta-catenin, decrease in beta-catenin and Snail (Sohn et al., 2019).
	The garcinol, which has an anti-cancer effect, increases phosphorylated beta-catenin, decreases beta-catenin and ZEB1/ZEB2, and inhibits EMT (Ahmad et al., 2012).
	The inhibition of sortilin by AF38469 (a sortilin inhibitor) or small interference RNA (siRNA) results in a decrease in beta-catenin and Twist expression in human glioblastoma cells (W. Yang et al., 2019).
	Histone deacetylase inhibitors affect EMT-related transcription factors including ZEB, Twist and Snail (Wawruszak et al., 2019).
	Snail and Zeb induces EMT and suppress E-cadherin (CDH1) (Batlle et al., 2000; Diaz et al., 2014; Peinado, Olmeda, & Cano, 2007).
KE3 => AO: Epithelial-mesenchymal transition (EMT) leads to human treatment-resistant gastric cancer	Empirical Support of the KE3 => AO is moderate.
	Rationale: EMT induces the expression of genes such as transporters of anti-cancer drug, which promotes anti-cancer drug resistance. EMT induces migration and metastasis of cancer cells, and share the phenotype of cancer stem cells, which is one of the hallmarks of human treatment-resistant gastric cancer (Tanabe et al., 2020a, 2020b).
	EMT activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in the resistance to doxorubicin (Saxena, Stephens, Pathak, & Rangarajan, 2011; Shibue & Weinberg, 2017).
	TGFbeta-1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties (Pirozzi et al., 2011; Shibue & Weinberg, 2017).
	Snail-induced EMT induces cancer metastasis and resistance to dendritic cell-mediated immunotherapy (Kudo-Saito et al., 2009).
	Zinc finger E-box-binding homeobox (ZEB1)-induced EMT results in the relief of 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 lymphocyte (CTL), subsequently the CD8+ T cell immunosuppression and metastasis (Chen et al., 2014).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Homo sapiens

Essentiality of the Key Events

Sustained ROS contributes into the initiation and development of human gastric cancer (Gu H. 2018).

Wnt signaling is involved in cancer malignancy (Tanabe, 2018).

Upon stimulation with Wnt ligand to Frizzled receptor, Wnt/beta-catenin signaling is activated. Wnt/beta-catenin consists of GSK3 beta inactivation, beta-catenin activation and up-regulation of transcription factors such as Zeb, Twist and Snail. The transcription factors Zeb, Twist and Snail relate to the activation of EMT-related genes. EMT is regulated with various gene networks (Tanabe, 2015c).

Weight of Evidence Summary

The Wnt signaling promotes EMT and cancer malignancy in colorectal cancer (Lazarova & Bordonaro, 2017). Although the potential pathways other than Wnt signaling exist in EMT induction and the mechanism underlaid cancer malignancy, Wnt signaling is one of the main pathways to induce EMT and cancer malignancy (Polakis, 2012).

Quantitative Consideration

Wnt signaling activates the CSCs to promote cancer malignancy (Reya & Clevers, 2005). The responses in KEs related to Wnt signaling, Frizzled activation, GSK3beta inactivation, beta-catenin activation, Snail, Zeb, Twist activation are dose-dependently related. The quantification of EMT and cancer malignancy would require the further investigation.

Considerations for Potential Applications of the AOP (optional)

AOP entitled “Chronic reactive oxygen species leading to human treatment-resistant gastric cancer” might be utilized for the development and risk assessment of anti-cancer drugs. EMT is involved in the acquisition of drug resistance, which is one of the critical features of cancer malignancy. The assessment of EMT would be the potential prediction of the adverse effects of anti-cancer drugs.

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Appendix 1

List of MIEs in this AOP

Event: 1753: Chronic reactive oxygen species

Short Name: Chronic ROS

Key Event Component

Process	Object	Action
response to reactive oxygen species	reactive oxygen species	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	MolecularInitiatingEvent

Stressors

Name
Ionizing Radiation
ferric nitrilotriacetate
Arsenic

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term

organ

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Reactive oxygen species (ROS) are generated through NADPH oxidases consisting of $p47^{phox}$ and $p67^{phox}$. Arsenic produces ROS [Zhang et al., 2011].

Ionizing radiation induces ROS [Kruk et al., 2017].

Iron(III)-nitrilotriacetate induces reactive oxygen species production via the transfer of an electron to molecular oxygen to form ROS [Tsuchiya et al., 2005, Akai et al., 2004].

Ionizing Radiation

Ionizing radiation induces reactive oxygen species.

(Ref. Reactive Oxygen and Nitrogen Species in Carcinogenesis: Implications of Oxidative Stress on the Progression and Development of Several Cancer Types

Author(s): Joanna Kruk, Hassan Y. Aboul-Enein*. Journal Name: Mini-Reviews in Medicinal Chemistry,

Volume 17 , Issue 11 , 2017, DOI : 10.2174/1389557517666170228115324)

ferric nitrilotriacetate

Iron(III)-nitrilotriacetate induces reactive oxygen species production via trasfer of an electron to molecular oxygen to form reactive oxygen species [Tsuchiya K, Akai K, Tokumura A, Abe S, Tamaki T, Takiguchi Y, Fukuzawa K. Biochim Biophys Acta. 2005 Aug 30;1725(1):111-9. doi: 10.1016/j.bbagen.2005.05.001, Akai K, Tsuchiya K, Tokumura A, Kogure K, Ueno S, Shibata A, Tamaki T, Fukuzawa K. Free Radic Res. 2004 Sep;38(9):951-62. doi: 10.1080/1071576042000261945.]

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Reactive oxygen species (ROS) are increased in human gastric cancer (*Homo sapiens*) [Gu et al., 2018].

Key Event Description

Reactive oxygen species (ROS) are radicals, ions, or molecules that have a single unpaired electron in their outermost shell of electrons, which can be categorized into two groups: free oxygen radicals and non-radical ROS [Liou et al., 2010]. Free oxygen radicals include superoxide ($O2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), nitric oxide ($NO^{\cdot}$), organic radicals ($R^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$), alkoxyl radicals ($RO^{\cdot}$), thiyl radicals ($RS^{\cdot}$), sulfonyl radicals ($ROS^{\cdot}$), thiyl peroxyl radicals ($RSOO^{\cdot}$), and disulfides (RSSR). Non-radical ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone/trioxygen (O_3), organic hydroperoxides (ROOH), hypochlorite (ClO^-), peroxynitrite ($ONOO^-$), nitrosoperoxycarbonate anion ($O{=}NOOCO_2^-$), nitrocarbonate anion ($O_2NOCO_2^-$), dinitrogen dioxide (N_2O_2), nitronium (NO_2^+), and highly reactive lipid- or carbohydrate-derived carbonyl compounds [Liou et al., 2010].

ROS are generated through NADPH oxidases consists of p47phox and p67phox. Arsenic produces ROS [Zhang et al., 2011]. The primary site of action for this event is DNA or proteins etc.

ROS play an important role in tumorigenesis [Zhang et al., 2011].

Chronic low-level increased ROS can alter the tumor microenvironment and promote cancer stem cell renewal, leading to therapeutic resistance [Gu et al., 2018].

The reason why this chronic ROS KE has been created is because it is important to have chronic ROS, but not just instant increased ROS, since ROS have a double-edged effect.

How it is Measured or Detected

Hydrogen peroxide (H_2O_2) can be detected with a colorimetric probe, which reacts with H_2O_2 in a 1:1 stoichiometry to produce a bright pink colored product, followed by the detection with a standard colorimetric microplate reader with a filter in the 540-570 nm range.

ROS in the blood can be detected using superparamagnetic iron oxide nanoparticles (SPION)-based biosensor [Lee et al., 2020].

ROS can be detected by fluorescent probes such as *p*-methoxy-phenol derivative [Ashoka et al., 2020].

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List of Key Events in the AOP

Event: 1940: Increases in cellular reactive oxygen species

Short Name: Increases in cellular ROS

Key Event Component

Process	Object	Action
reactive oxygen species biosynthetic process	reactive oxygen species	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	KeyEvent

Stressors

Name
Ionizing Radiation
ferric nitrilotriacetate
Arsenic
Heavy metals (cadmium, lead, copper, iron, nickel)
Mitochondrial ETC inhibitors
Potassium bromate

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI
human and other cells in culture	human and other cells in culture	Moderate	NCBI
mouse	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

This KE is broadly applicable across species.

Key Event Description

Reactive oxygen species (ROS) refers to chemical species superoxide, hydrogen peroxide, and their secondary reactive products. In the biological context, ROS are signaling molecules with important roles in cell energy metabolism, cell proliferation and fate. Therefore, balancing ROS levels at the cellular and tissue level is an important part of many biological processes. Disbalance, mainly increase of ROS levels, can cause cell dysfunction and irreversible cell damage.

ROS are produced from both exogenous stressors and normal endogenous cellular processes, such as the mitochondrial electron transport chain (ETC). Inhibition of the ETC can result in the accumulation of ROS. Exposure to chemicals, heavy metal ions, or ionizing radiation can also result in increased production of ROS. Chemicals and heavy metal ions can deplete cellular antioxidants reducing the cell's ability to control cellular ROS and resulting in the accumulation of ROS. Cellular antioxidants include glutathione (GSH), protein sulfhydryl groups, superoxide dismutase (SOD).

ROS are radicals, ions, or molecules that have a single unpaired electron in their outermost shell of electrons, which can be categorized into two groups: free oxygen radicals and non-radical ROS [Liou et al., 2010].

<Free oxygen radicals>

superoxide	$O_2\cdot^-$
hydroxyl radical	·OH
nitric oxide	NO·
organic radicals	R·
peroxyl radicals	ROO·
alkoxyl radicals	RO·
thiyl radicals	RS·
sulfonyl radicals	ROS·
thiyl peroxyl radicals	RSOO·
disulfides	RSSR

<Non-radical ROS>

hydrogen peroxide	H_2O_2
singlet oxygen	$^1O_2$
ozone/trioxygen	O_3
organic hydroperoxides	ROOH
hypochlorite	ClO^-
peroxynitrite	$ONOO^-$
nitrosoperoxycarbonate anion	$O{=}NOOCO_2^-$
nitrocarbonate anion	$O_2NOCO_2^-$
dinitrogen dioxide	N_2O_2
nitronium	NO_2^+
highly reactive lipid- or carbohydrate-derived carbonyl compounds	

Potential sources of ROS include NADPH oxidase, xanthine oxidase, mitochondria, nitric oxide synthase, cytochrome P450, lipoxygenase/cyclooxygenase, and monoamine oxidase [Granger, et al., 2015]. ROS are generated through NADPH oxidases consisting of $p47^{phox}$ and $p67^{phox}$. ROS are generated through xanthine oxidase activation in sepsis [Ramos, et al., 2018]. Arsenic produces ROS [Zhang et al., 2011]. Mitochondria-targeted paraquat and metformin mediate ROS production [Chowdhury, et al., 2020]. ROS are generated by bleomycin [Lu, et al., 2010]. Radiation induces dose-dependent ROS production [Ji, et al., 2019].

ROS are generated in the course of cellular respiration, metabolism, cell signaling, and inflammation [Dickinson and Chang 2011; Egea, et al. 2017]. Hydrogen peroxide is also made by the endoplasmic reticulum in the course of protein folding. Nitric oxide (NO) is produced at the highest levels by nitric oxide synthase in endothelial cells and phagocytes. NO production is one of the main mechanisms by which phagocytes kill bacteria [Wang et al., 2017]. The other species are produced by reactions with superoxide or peroxide, or by other free radicals or enzymes.

ROS activity is principally local. Most ROS have short half-lives, ranging from nano- to milliseconds, so diffusion is limited, while reactive nitrogen species (RNS) nitric oxide or peroxynitrite can survive long enough to diffuse across membranes [Calcerrada, et al. 2011]. Consequently, local concentrations of ROS are much higher than average cellular concentrations, and signaling is typically controlled by colocalization with redox buffers [Dickinson and Chang 2011; Egea, et al. 2017].

Although their existence is limited temporally and spatially, ROS interact with other ROS or with other nearby molecules to produce more ROS and participate in a feedback loop to amplify the ROS signal, which can increase RNS. Both ROS and RNS also move into neighboring cells and ROS can increase intracellular ROS signaling in neighboring cells [Egea, et al. 2017].

How it is Measured or Detected

<Direct detection>

Many fluorescent compounds can be used to detect ROS, some of which are specific and others are less specific.

ROS can be detected by fluorescent probes such as *p*-methoxy-phenol derivative [Ashoka et al., 2020].

Chemiluminescence analysis can detect the superoxide, where some probes have a wider range for detecting hydroxyl radical, hydrogen peroxide, and peroxynitrite [Fuloria et al., 2021].

ROS in the blood can be detected using superparamagnetic iron oxide nanoparticles (SPION)-based biosensor [Lee et al., 2020].

Hydrogen peroxide (H_2O_2) can be detected with a colorimetric probe, which reacts with H_2O_2 in a 1:1 stoichiometry to produce a bright pink colored product, followed by the detection with a standard colorimetric microplate reader with a filter in the 540-570 nm range.

The levels of ROS can be quantified using multiple-step amperometry using a stainless steel counter electrode and non-leak Ag|AgCl reference node [Flaherty et al., 2017].

Singlet oxygen can be measured by monitoring the bleaching of *p*-nitrosodimethylaniline at 440 nm using a spectrophotometer with imidazole as a selective acceptor of singlet oxygen [Onoue et al., 2014].

<Indirect Detection>

Alternative methods involve the detection of redox-dependent changes to cellular constituents such as proteins, DNA, lipids, or glutathione [Dickinson and Chang 2011; Wang, et al. 2013; Griendling, et al. 2016]. However, these methods cannot generally distinguish between the oxidative species behind the changes, and cannot provide good resolution for kinetics of oxidative activity.

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Event: 1754: Sustained tissue damage / macrophage activation / porcupine-induced Wnt secretion

Short Name: Sustained tissue damage, macrophage activation and Wnt secretion

Key Event Component

Process	Object	Action
Wnt protein secretion	protein-serine O-palmitoleoyltransferase porcupine	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	KeyEvent

Stressors

Name
Radiation

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

Radiation

Radiation induces porcupine-induced Wnt secretion in macrophage (Saha et al., 2016a).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Oligomerization of FZD and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) activates Wnt/beta-catenin signaling in *Homo sapiens* (Hua et al., 2018).

Key Event Description

Porcupine, which is a trans-membrane endoplasmic reticulum O-acyl transferase, is important for the secretion of Wnt ligands(Saha et al., 2016a). WNTs are secreted proteins that contain 22-24 conserved cysteine residues (Foulquier et al., 2018). The WNT molecules consist of molecular families including WNT1, WNT2, WNT2B/WNT13, WNT3, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT10B, WNT11, and WNT16. (Clevers & Nusse, 2012; M. Katoh, 2001; Kusserow et al., 2005)

Wnt proteins consist of 350-400 amino acids (Saito-Diaz et al., 2013).

WNT ligands are known to trigger at least three different downstream signaling cascades including canonical WNT/beta-catenin signaling pathway, non-canonical WNT/Ca^{2+} pathway, and planer cell polarity (PCP) pathway(De, 2011; Lai, Chien, & Moon, 2009; Willert & Nusse, 2012). WNTs bind to Frizzled proteins, which are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (Bhanot et al., 1996; Clevers, 2006). Wnt signaling begins with the binding of Wnt ligand towards the Frizzled receptors (Mohammed et al., 2016).

Wnt ligands bind to Frizzled (FZD) receptors which are seven transmembrane-domain protein receptors (Nile, Mukund, Stanger, Wang, & Hannoush, 2017). At least 10 FZD receptors are identified in human cells. FZD receptor is activated by Wnt ligand binding (MacDonald, Tamai, & He, 2009).

How it is Measured or Detected

- Secretion of WNT requires a number of other dedicated factors including the sortin receptor Wntless (WLS), which binds to Wnt and escorts it to the cell surface (Banziger et al., 2006; Ching & Nusse, 2006)
- Wnt signaling is activated by the gene mutations of the signaling components (Ziv et al., 2017).
- Wnt1, Wnt3a, and Wnt5a protein expression are measured by immunoblotting using antibodies for Wnt1, Wnt3a, and Wnt5a, respectively (J. Du et al., 2016; B. Wang et al., 2017).
- WNT2, of which expression is detected by quantitative PCR, immunoblotting, and immunohistochemistry, induces EMT (Zhou et al., 2016).
- Frizzled receptor protein level on the cell surface is measured by flow cytometry with pan-FZD antibody (Jiang et al., 2015; Zeng et al., 2018). DVL protein level is measured by immunoblotting with anti-DVL2 antibodies (Zeng et al., 2018).
- Fzd mRNA level is measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Zeng et al., 2018).
- The up-regulation of WNT ligand expression occurs in *Homo sapiens* (B. Wang et al., 2017).
- The Wnt genes play an important role in the secretion from cells, glycosylation, and tight association with the cell surface and extracellular matrix in *Drosophila melanogaster* (Willert & Nusse, 2012).

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Event: 1755: Proliferation / beta-catenin activation

Short Name: Proliferation / beta-catenin activation

Key Event Component

Process	Object	Action
regulation of beta-catenin-TCF complex assembly	beta-catenin-TCF complex	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Beta-catenin is stabilized and translocated into nucleus in *Homo sapiens* (Huang et al., 2019).

Beta-catenin is activated in *Homo sapiens* (Huang et al., 2019) (Naujok et al., 2014).

Key Event Description

Upon the Wnt signaling activation, beta-catenin is stabilized and activated via inhibition of the phosphorylation by GSK3beta (Huang et al., 2019). Once the beta-catenin is stabilized, it translocates into the nucleus and enhances the expression of target genes of Wnt/beta-catenin signaling pathway (Huang et al., 2019). Beta-catenin activation is related to cancer (Tanabe, 2014).

Dishevelled (DVL), a positive regulator of Wnt signaling, forms the complex with FZD and leads to trigger the Wnt signaling together with Wnt coreceptor low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (Clevers & Nusse, 2012**;** Jiang, et al., 2015**). DVL, however, has a controversial role to promote Wnt receptor degradation (**Jiang et al., 2015**). Meanwhile, DVL-dependent regulation of FZD level is involved in mTORC1 signaling suppression via Wnt/beta-catenin signaling (**Zeng et al., 2018**). The recruitment of Axin to the DVL-FZD complex induces the beta-catenin stabilization and activation. The stabilized beta-catenin translocates into the nucleus, which forms the complex with TCF to induce the up-regulated expression of proliferation-related genes.**

How it is Measured or Detected

The beta-catenin level in nucleus is measured by immunoblotting with anti-beta-catenin antibody (Huang et al., 2019).

The beta-catenin nuclear translocation is measured by immunofluorescence assay (Huang et al., 2019).

Activity of beta-catenin is measured by Wnt/beta-catenin activity assay, in which the vector containing the firefly luciferase gene controlled by TCF/LEF binding sites is transfected in the cells (Naujok et al., 2014).

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Event: 1650: Epithelial-mesenchymal transition

Short Name: Epithelial-mesenchymal transition

Key Event Component

Process	Object	Action
epithelial to mesenchymal transition	cellular_component	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent

Stressors

Name
GOLPH3
LiCl
D-2-hydroxyglutarate

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

GOLPH3

GOLPH3 induces EMT (Sun et al., 2017).

LiCl

LiCl induces EMT (Fang et al., 2018).

D-2-hydroxyglutarate

D-2-hydroxyglutarate induces EMT (Colvin et al., 2016).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Wnt5a expression leads to epithelial-mesenchymal transition (EMT) and metastasis in non-small-cell lung cancer in *Homo sapiens* (Wang et al., 2017).
- WNT2 expression lead to EMT induction in *Homo sapiens* (Zhou et al., 2016).
- EMT is induced in cancer and involved in cancer metastasis in *Homo sapiens* (Suarez-Carmona, Lesage, Cataldo, & Gilles, 2017) (Du & Shim, 2016).

Key Event Description

Epithelial-mesenchymal transition (EMT) is a phenomenon in which the cells transit from epithelial-like into mesenchymal-like phenotypes (Huan et al., 2022; Tanabe, 2017; Tanabe et al., 2015). In cancer, cells exhibiting EMT features contribute to metastasis and drug resistance.

It is known that D-2-hydroxyglurate induces EMT (Guerra et al., 2017; Jia et al., 2018; Mishra et al., 2018; Sciacovelli & Frezza, 2017). D-2-hydroxyglurate, an inhibitor of Jumonji-family histone demethylase, increased the trimethylation of histone H3 lysine 4 (H3K4) in the promoter region of the zinc finger E-box-binding homeobox 1 (ZEB1), followed by the induction of EMT (Colvin et al., 2016).

Wnt5a induces EMT and metastasis in non-small-cell lung cancer (Wang et al., 2017).

EMT is related to Wnt/beta-catenin signaling and is important for treatment-resistant cancer (Tanabe et al., 2016)

TGFbeta induces EMT (Wendt et al., 2010).

ZEB is one of the critical transcription factors for EMT regulation (Zhang et al., 2015).

SNAI1 (Snail) is an important transcription factor for cell differentiation and survival. The phosphorylation and nuclear localization of Snail1 induced by Wnt signaling pathways are critical for the regulation of EMT (Kaufhold & Bonavida, 2014).

Transcription factors SNAI1 and TWIST1 induce EMT (Hodge et al., 2018) (Mani et al., 2008)

It is suggested that Sp1, a transcription factor involved in cell growth and metastasis, is induced by cytochrome P450 1B1 (CYP1B1), and promotes EMT, which leads to cell proliferation and metastasis (Kwon et al., 2016).

How it is Measured or Detected

- EMT can be detected by immunostaining with pro-surfactant protein-C (pro-SPC) and N-cadherin in idiopathic pulmonary fibrosis (IPF) lung *in vivo* (Kim et al., 2006).
- EMT can be detected by immunostaining with vimentin in lung alveola *in vivo* (Kim et al., 2006).
- EMT can be detected as the increased level of the transcription factors, zinc finger E-box-binding homeobox (ZEB), Twist and

Snail (Huang et al., 2022).

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List of Adverse Outcomes in this AOP

Event: 1651: Treatment-resistant gastric cancer

Short Name: Resistant gastric cancer

Key Event Component

Process	Object	Action
regulation of cellular response to drug		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	AdverseOutcome

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Drug resistance occurs in *Homo sapiens* (Du & Shim, 2016).

Key Event Description

It is known that diffuse-type gastric cancer, which has a poor prognosis, is treatment-resistant and more malignant compared to intestinal-type gastric cancer (Tanabe et al., 2014). Drug resistance is involved in EMT, which is an important phenomenon exhibiting features similar to cancer stem cells (CSCs) (Du & Shim, 2016).

EMT is involved in metastasis and cancer therapy resistance (Smith & Bhowmick, 2016).

How it is Measured or Detected

Treatment-resistant gastric cancer and EMT can be detected with biomarkers (Zeisberg & Neilson, 2009).

Treatment-resistant gastric cancer which exhibits EMT phenotype can be detected as the increased level of the transcription factors, zinc finger E-box-binding homeobox 1/2 (ZEB1/2), SNAI1/2, and TWIST2 which are associated with the activation of EMT-related genes (Tanabe et al., 2022a and 2022b).

Regulatory Significance of the AO

Drug resistance is very important in cancer treatment since cancer metastasis and recurrence are some of the main obstacles to treating cancer. Cancer stem cells that share the phenotype of EMT may be targeted in anti-cancer drug development.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2069: Chronic ROS leads to Sustained tissue damage, macrophage activation and Wnt secretion

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Prolonged ROS induces inflammation and tissue damage in *Homo sapiens* (Vallée & Lecarpentier, 2018).

Key Event Relationship Description

ROS production causes tissue damage (Gao, Zhou, Lin, Paus, & Yue, 2019). ROS production is involved in Wnt-driven tumorigenesis (Myant et al., 2013). The prolonged ROS induces inflammation leading to carcinogenesis (Vallée & Lecarpentier, 2018).

Injury causes the Porcupine-induced Wnt secretion (Saha et al., 2016).

Evidence Supporting this KER

Biological Plausibility

Sustained ROS increase caused by/causes DNA damage, which will alter several signaling pathways including Wnt signaling. Macrophages accumulate into injured tissue to recover the tissue damage, which may be followed by porcupine-induced Wnt secretion. ROS stimulate inflammatory factor production and Wnt/beta-catenin signaling (Vallée & Lecarpentier, 2018).

Empirical Evidence

Incidence concordance

Production of ROS by DNA double-strand break causes tissue damages (Gao et al., 2019).

ROS signaling induces Wnt/beta-catenin signaling (Pérez, Taléns-Visconti, Rius-Pérez, Finamor, & Sastre, 2017).

Uncertainties and Inconsistencies

The balance of ROS signaling is important, and dual effects of ROS should be taken in consideration. The ROS may enhance Wnt/beta-catenin proliferating pathways to promote tumorigenesis, while ROS may disrupt tumor progression by different pro-apoptotic mechanisms (Pérez et al., 2017). It is also known that Wnt signaling induces ROS signaling (Cheung et al., 2016). Wnt/beta-catenin signaling control by ROS needs to be further investigated (Caliceti, Nigro, Rizzo, & Ferrari, 2014).

Quantitative Understanding of the Linkage

Response-response relationship

ROS induces inflammatory responses (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Oxidant induces ROS generation and p38 MAPK activation in macrophages (Conway & Kinter, 2006). ROS induce tissue damage in cardiac myocytes (Miller & Cheung, 2016; Yang et al., 2006).

Time-scale

For the colony formation assay, cells were treated with 400 microM/L H_2O_2 for 1 week, where the medium was changed every three days (Wang et al., 2019).

Known modulating factors

GPX2, an activator of Wnt/beta-catenin signaling, is identified as a key regulator of intracellular H_2O_2 levels and an inhibitor of apoptosis (Wang et al., 2019).

Known Feedforward/Feedback loops influencing this KER

The reduction in ROS levels in the human serum albumin-treated cerebral ischemia/reperfusion-induced injury may be mediated by Wnt/beta-catenin signaling (Tang, Shen, Zhang, Yang, & Liu, 2019).

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Relationship: 2526: Increases in cellular ROS leads to Sustained tissue damage, macrophage activation and Wnt secretion

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Prolonged ROS induces inflammation and tissue damage in *Homo sapiens* (Vallée & Lecarpentier, 2018).

Key Event Relationship Description

ROS production causes tissue damage (Gao, Zhou, Lin, Paus, & Yue, 2019). ROS production is involved in Wnt-driven tumorigenesis (Myant et al., 2013).

Evidence Supporting this KER

Biological Plausibility

Sustained ROS increase caused by/causes DNA damage, which will alter several signaling pathways including Wnt signaling.

Macrophages accumulate into injured tissue to recover the tissue damage, which may be followed by porcupine-induced Wnt secretion. ROS stimulate inflammatory factor production and Wnt/beta-catenin signaling (Vallée & Lecarpentier, 2018).

Empirical Evidence

Production of ROS by DNA double-strand break causes tissue damages (Gao et al., 2019).

ROS signaling induces Wnt/beta-catenin signaling (Pérez, Taléns-Visconti, Rius-Pérez, Finamor, & Sastre, 2017).

Uncertainties and Inconsistencies

The balance of ROS signaling is important, and dual effects of ROS should be taken in consideration. The ROS may enhance Wnt/beta-catenin proliferating pathways to promote tumorigenesis, while ROS may disrupt tumor progression by different pro-apoptotic mechanisms (Pérez et al., 2017). It is also known that Wnt signaling induces ROS signaling (Cheung et al., 2016). Wnt/beta-catenin signaling control by ROS needs to be further investigated (Caliceti, Nigro, Rizzo, & Ferrari, 2014).

Quantitative Understanding of the Linkage

Response-response relationship

ROS induces inflammatory responses (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Oxidant induces ROS generation and p38 MAPK activation in macrophages (Conway & Kinter, 2006). ROS induce tissue damage in cardiac myocytes (Miller & Cheung, 2016; Yang et al., 2006).

Time-scale

For the colony formation assay, cells were treated with 400 microM/L H2O2 for 1 week, where the medium was changed every three days (Wang et al., 2019).

Known modulating factors

GPX2, an activator of Wnt/beta-catenin signaling, is identified as a key regulator of intracellular H2O2 levels and an inhibitor of apoptosis (Wang et al., 2019).

Known Feedforward/Feedback loops influencing this KER

The reduction in ROS levels in the human serum albumin-treated cerebral ischemia/reperfusion-induced injury may be mediated by Wnt/betacatenin signaling (Tang, Shen, Zhang, Yang, & Liu, 2019).

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Yang, K. T., Chang, W. L., Yang, P. C., Chien, C. L., Lai, M. S., Su, M. J., & Wu, M. L. (2006). Activation of the transient receptor potential M2 channel and poly(ADP-ribose) polymerase is involved in oxidative stress-induced cardiomyocyte death. Cell Death & Differentiation, 13(10), 1815-1826. doi:10.1038/sj.cdd.4401813

Relationship: 2070: Sustained tissue damage, macrophage activation and Wnt secretion leads to Proliferation / beta-catenin activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Wnt/beta-catenin signaling, which regulates key cellular functions including proliferation, is a highly conserved pathway through evolution (Pai et al., 2017).

Key Event Relationship Description

Secreted Wnt ligand stimulates Wnt/beta-catenin signaling, in which beta-catenin is activated. Wnt ligand binds to Frizzled receptor, which leads to GSK3beta inactivation. GSK3beta inactivation leads to beta-catenin dephosphorylation, which avoids the ubiquitination of the beta-catenin and stabilizes the beta-catenin (Clevers & Nusse, 2012). The translocation of stabilized beta-catenin induces the transcription of genes involved in proliferation (Pai et al., 2017).

Evidence Supporting this KER

Biological Plausibility

Canonical Wnt pathway consists of Wnt, GSK3beta, and beta-catenin cascade (Clevers & Nusse, 2012; Hatsell, Rowlands, Hiremath, & Cowin, 2003).

GSK3beta recruitment to LRP6 leads to form un-phosphorylated beta-catenin inducing the stabilization and translocation of the beta-catenin (MacDonald, Tamai, & He, 2009).

Stabilized beta-catenin accumulates in cytosol and translocates into the nucleus leading to beta-catenin activation (MacDonald et al., 2009).

Empirical Evidence

[Incidence concordance]

Dishevelled (DVL), a positive regulator of Wnt signaling, form the complex with FZD and lead to trigger the Wnt signaling together with Wnt coreceptor low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (Clevers & Nusse, 2012; Jiang, Charlat, Zamponi, Yang, & Cong, 2015). Wnt binds to FZD and activates the Wnt signaling (Clevers & Nusse, 2012; Janda, Waghray, Levin, Thomas, & Garcia, 2012; Nile, Mukund, Stanger, Wang, & Hannoush, 2017). Wnt binding towards FZD induces the formation of the protein complex with LRP5/6 and DVL, leading to the downstream signaling activation including beta-catenin (Clevers & Nusse, 2012).

Uncertainties and Inconsistencies

Some Wnt ligands bind to FZD, leading to Wnt/beta-catenin signaling inactivation. DVL, a positive regulator of Wnt signaling, has a controversial role to promote Wnt receptor degradation (Jiang et al., 2015). DVL-dependent regulation of FZD level is involved in mTORC1 signaling suppression via Wnt/beta-catenin signaling (Zeng et al., 2018)

GSK3beta phosphorylates LRP6 as well as remaining GSK3 beta phosphorylates beta-catenin which would be ubiquitinated and degradated (MacDonald et al., 2009).

Quantitative Understanding of the Linkage

Response-response relationship

Wnt3 promotes proliferation and survival in HUVECs (Shen et al., 2018).

GSK3beta inhibition by 1 uM of SB216763 or 5 uM of BRD3731 results in the decreased phosphorylation and stabilization of beta-catenin (Stump et al., 2019). The level of beta-catenin is increased by the inhibition of GSK3beta kinase activity (Stump et al., 2019). GSK3beta inhibition by small interference RNA (siRNA) of GSK3beta results in the decreased phosphorylation and increased expression of beta-catenin (Stump et al., 2019).

Time-scale

FZD7 enhances the activity of canonical Wnt/beta-catenin signaling with the treatment of WNT3A for 1 to 6 hrs (Cao et al., 2017). The treatment with SB216763 or BRD3731, GSK3beta inhibitors, decreases phosphorylated beta-catenin and increased beta-catenin expression in 48 hours (Stump et al., 2019). The cells are treated with GSK3beta small interference RNA (siRNA) for 48 hours to silence the expression of GSK3beta, which results in the activation of beta-catenin pathway (Stump et al., 2019).

Known modulating factors

FZD5 can activate WNT3A/beta-catenin signaling in a dose-dependent manner (Hua et al., 2018). The increase in FZD5 protein enhances cell response to WNT3A. (Hua et al., 2018). LRP5 can augment WNT3A/beta-catenin signaling in a dose-dependent manner (Hua et al., 2018). The binding of Wnt and FZD induce the formation of the protein complex with the Dvl, Axin, CK1 GSK3, beta-catenin and APC to induce the beta-catenin translocation into the nucleus (Clevers & Nusse, 2012).

Known Feedforward/Feedback loops influencing this KER

Beta-catenin is required and sufficient for the sequestration of GSK3 in acidic cytoplasmic endosomes (Taelman et al., 2010). Beta-catenin, of which level increases in Wnt signaling, facilitates GSK3 sequestration leading to feed-forward loop formation (Taelman et al., 2010). The Wnt ligand is antagonized with secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF), both of which can bind Wnts and inhibit interactions between WNT and FZD (Bovolenta, Esteve, Ruiz, Cisneros, & Lopez-Rios, 2008; Clevers & Nusse, 2012). The Dickkopf 1 (DKK1) can disrupts Wnt-induced FZD-LRP6 complex formation (Clevers & Nusse, 2012; Ellwanger et al., 2008; Semenov, Zhang, & He, 2008).

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Relationship: 2071: Proliferation / beta-catenin activation leads to Epithelial-mesenchymal transition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- The inhibition of c-MET decreases the expression of beta-catenin and Snail in human diffuse-type gastric cancer (*Homo sapiens*) (Sohn et al., 2019).
- The treatment with garcinol decreases the expression of beta-catenin and ZEB1/ZEB2 in human breast cancer cells (*Homo sapiens*) (Ahmad et al., 2012).
- Zeb1 activation leads to EMT via Prex1 activation in NCH421k, NCH441, and NCH644 human glioblastoma model cells (*Homo sapien*s) (Rosmaninho et al., 2018).
- Zeb1 siRNA induced the suppression of EMT in SGC-7901 human gastric cancer cell line (*Homo sapiens*) (Xue et al., 2019). Snail induces EMT in SAS and HSC-4 human head and neck squamous cancer cells (*Homo sapiens*) (Ota et al., 2016).
- Snail induces EMT in B16-F10 murine melanoma cells (*Mus musculus*) (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009; Wang, Shi, Chai, Ying, & Zhou, 2013).
- Twist1 is related to EMT in MCF-7 and MDA-MB-231 human breast cancer cell lines (*Homo sapiens*) (Menendez-Menendez et al., 2019).
- Twist induces EMT in Huh7 human hepatocellular carcinoma cell lines (*Homo sapiens*) (Hu et al., 2019).

Key Event Relationship Description

Beta-catenin activation, of which mechanism include the stabilization of the dephosphorylated beta-catenin and translocation of beta-catenin into the nucleus, induce the formation of beta-catenin-TCF complex and transcription of transcription factors such as Snail, Zeb and Twist (Clevers & Nusse, 2012) (Ahmad et al., 2012; Pearlman, Montes de Oca, Pal, & Afaq, 2017; Sohn et al., 2019;

Yang et al., 2019).

EMT-related transcription factors including Snail, ZEB and Twist are up-regulated in cancer cells (Diaz, Vinas-Castells, & Garcia de Herreros, 2014). The transcription factors such as Snail, ZEB and Twist bind to E-cadherin (CDH1) promoter and inhibit the CDH1 transcription via the consensus E-boxes (5'-CACCTG-3' or 5'-CAGGTG-3'), which leads to EMT (Diaz et al., 2014).

Evidence Supporting this KER

Biological Plausibility

The treatment of human gastric cancer cells with INC280, which inhibits c-MET overexpressed in diffuse-type gastric cancer with poor prognosis, shows downregulation in beta-catenin and Snail expression,(Sohn et al., 2019).

The treatment with garcinol, a polyisoprenylated benzophenone derivative that is obtained from *Garcinia indica* extract, induced ZEB1 and ZEB2 down-regulation, increase in phosphorylated beta-catenin, and decrease in nuclear beta-catenin in human breast cancer cells (Ahmad et al., 2012).

Sortilin, a member of the Vps10p sorting receptor family which is highly expressed in high-grade malignant glioma, positively regulates GSK-3beta/beta-catenin/Twist signaling pathway in glioblastoma (Yang et al., 2019).

TM4SF1 promotes EMT via Wnt/beta-catenin/SOX2 pathway in colorectal cancer (Yang et al., 2020).

The transcription factors such as Snail, Zeb, and Twist inhibit the CDH1 expression through their binding towards the promoter of CDH1, which leads to inhibition of cell adhesion and EMT (Diaz et al., 2014)

Empirical Evidence

[Dose concordance]

The inhibition of sortilin by AF38469 (a sortilin inhibitor) or small interference RNA (siRNA) results in a decrease in beta-catenin and Twist expression in human glioblastoma cells (Yang et al., 2019).

[Time concordance]

The complex of beta-catenin and TCF4 induces epithelial-mesenchymal transition (EMT)-activator ZEB (Sanchez-Tillo E et al., 2011).

[Incidence concordance]

The inhibition of c-MET, which is overexpressed in diffuse-type gastric cancer, induced an increase in phosphorylated beta-catenin, decrease in beta-catenin and Snail (Sohn et al., 2019).

The garcinol, which has an anti-cancer effect, increases phosphorylated beta-catenin, decreases beta-catenin and ZEB1/ZEB2, and inhibits EMT (Ahmad et al., 2012).

Histone deacetylase inhibitors affect EMT-related transcription factors including ZEB, Twist, and Snail (Wawruszak et al., 2019).

Snail and Zeb induces EMT and suppress E-cadherin (CDH1) (Batlle et al., 2000; Diaz et al., 2014; Peinado, Olmeda, & Cano, 2007).

Uncertainties and Inconsistencies

It is possible that the inhibition of ZEB1 and ZEB2 by garcinol treatment is caused by down-regulation of NFkappaB and Wnt/beta-catenin signaling (Ahmad et al., 2012).

The EMT is induced different transcription factors other than Zeb, Twist, and Snail, which includes E47 and KLF8 (Diaz et al., 2014).

Zeb, Twist, and Snail may activate or inactivate different genes or molecules to induce phenomena related to EMT and other phenomena other than EMT (Li & Balazsi, 2018).

Quantitative Understanding of the Linkage

Response-response relationship

The treatment with AF38469, a sortilin inhibitor, in 0, 100, 200, 400, 800, and 1600 nM concentration inhibited beta-catenin and Twist (EMT regulator) expression dose-dependently in human glioblastoma cells (Yang et al., 2019).

Snail (SNAI1, a key transcription factor of EMT induced by beta-catenin) mRNA is methylated, and N^6-methyladenosine (m^6A) in its coding region (CDS) and 3' untranslated region (3'UTR) are significantly enriched during EMT progression (Lin et al., 2019). The m^6A enrichment fold of *SNAI1* mRNA in EMT cells is about 2.3-fold greater than in control cells (Lin et al., 2019).

Time-scale

Nuclear accumulation of beta-catenin induces endogenous ZEB1 in 15 and 30 min (Sanchez-Tillo E et al., 2011).

The treatment with 25 uM of garcinol for 48 hours induced an increase in phosphorylated beta-catenin and decreased nuclear beta-catenin protein and ZEB1/ZEB2 mRNA in human breast cancer cells (Ahmad et al., 2012).

The treatment with AF38469, a sortilin inhibitor, for 0, 2, 4, 8, 16, or 24 hours shows that the expression of beta-catenin and Twist decrease in 8 hours followed by the subsequent decrease in 16 and 24 hours in human glioblastoma cells (Yang et al., 2019).

Snail (SNAI1) transfection for 48 hours induces the repression of E-cadherin (CDH1) protein expression (Lin et al., 2019).

SNAI1 mRNA in polysome is up-regulated in EMT-undergoing HeLa cells treated with 10 ng/ml of TGF-beta for 3 days compared with control cells (Lin et al., 2019).

Known modulating factors

The proto-oncogene MET regulates beta-catenin and Snail expression (Sohn et al., 2019).

The inhibition of GSK3beta by SB216763 induced expression of beta-catenin and Twist, as well as mesenchymal markers such as N-cadherin, vimentin, and MMP9 (Yang et al., 2019).

The decrease in E-cadherin (CDH1), a cell adhesion molecule, is related to EMT (Diaz et al., 2014).

Methyltransferase-like 3 (METTL3) modulates methylation of Snail (SNAI1) mRNA and EMT (Lin et al., 2019).

The binding of beta-catenin to members of the TCF/LEF family transcription factors increase gene expression related to EMT such as Twist and decrease E-cadherin protein expression (Qualtrough, Rees, Speight, Williams, & Paraskeva, 2015).

Known Feedforward/Feedback loops influencing this KER

The inhibited expression of phosphorylated GSK3beta, beta-catenin, and Twist by sortilin inhibition is reversed by GSK3beta inhibition. Furthermore, twist overexpression by lentivirus increased the inhibited expression of N-cadherin, MMP9, and vimentin and reverses the inhibitory effect of AF38469 on sortilin, which suggests that sortilin induces glioblastoma invasion mainly via GSK3beta/beta-catenin/Twist induced mesenchymal transition (Yang et al., 2019).

The inhibition of Hedgehog signaling pathway with cyclopamine reduces beta-catenin-TCF transcriptional activity, decreases the Twist expression, induces E-cadherin expression, and inhibits EMT (Qualtrough et al., 2015).

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Relationship: 1929: Epithelial-mesenchymal transition leads to Resistant gastric cancer

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Chronic reactive oxygen species leading to human treatment-resistant gastric cancer	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	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

Some population of the cells exhibiting EMT demonstrates the feature of cancer stem cells (CSCs), which are related to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015).

EMT phenomenon is related to cancer metastasis and cancer therapy resistance (Smith & Bhowmick, 2016; Tanabe, 2013). The increased expression of enzymes that degrade the extracellular matrix components and the decrease in adhesion to the basement membrane in EMT induces the cell to escape from the basement membrane and metastasis (Smith & Bhowmick, 2016). Morphological changes observed during EMT are associated with therapy resistance (Smith & Bhowmick, 2016).

Evidence Supporting this KER

Biological Plausibility

The morphological and physiological changes associated with EMT are involved in invasiveness and drug resistance (Shibue & Weinberg, 2017). The EMT-activated particular carcinoma cells in primary tumors invade the surrounding stroma (Shibue & Weinberg, 2017). The EMT –activated carcinoma cells interact with the surrounding extracellular matrix protein to induce focal adhesion kinase and extracellular signal-related kinase activation, followed by the transforming growth factor-beta (TGFbeta) and canonical and/or noncanonical Wnt pathways to induce cancer stem cell (CSC) properties which contribute to the drug resistance (Shibue & Weinberg, 2017).

EMT-associated down-regulation of multiple apoptotic signaling pathways induces drug efflux and slows cell proliferation to induce the general resistance of carcinoma cells to anti-cancer drugs (Shibue & Weinberg, 2017).

Snail, an EMT-related transcription factor, induces the expression of the AXL receptor tyrosine kinase, which enables the cancer cells to survive by the activation of AXL signaling triggered by the binding of its ligand growth arrest-specific protein 6 (GAS6) (Shibue & Weinberg, 2017).

The EMT-activated cells evade the lethal effect of cytotoxic T cells, which include the elevated expression of programmed cell death 1 ligand (PD-L1) which binds to the programmed cell death protein 1 (PD-1) inhibitory immune-checkpoint receptor on the cell surface of cytotoxic T cells (Shibue & Weinberg, 2017).

Empirical Evidence

Incidence concordance

Slug/Snai2, a *ces*-1-related zinc finger transcription factor gene, confers resistance to p53-mediated apoptosis of hematopoietic progenitors by repressing *PUMA* (also known as *BBC3*, encoding Bcl-2-binding component 3) (Inukai et al., 1999; Shibue & Weinberg, 2017; W.-S. Wu et al., 2005).

EMT activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in the resistance to doxorubicin (Saxena, Stephens, Pathak, & Rangarajan, 2011; Shibue & Weinberg, 2017)

TGFbeta-1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties (Pirozzi et al., 2011; Shibue & Weinberg, 2017).

Snail-induced EMT induces cancer metastasis and resistance to dendritic cell-mediated immunotherapy (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009).

Zinc finger E-box-binding homeobox (ZEB1)-induced EMT results in the relief of 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 lymphocyte (CTL), subsequently the $CD8^+$ T cell immunosuppression and metastasis (Chen et al., 2014).

Uncertainties and Inconsistencies

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).

Quantitative Understanding of the Linkage

Response-response relationship

Induction of EMT by TGFbeta and Twist increases the gene expression of EMT markers such as Snail, Vimentin, N-cadherin, and ABC transporters including ABCA3, ABCC1, ABCC3, and ABCC10 (Saxena et al., 2011).

Human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrates the increased cell viability compared to control HMLE in the treatment with about 0.3, 3, 30 mM of doxorubicin, dose-dependently (Saxena et al., 2011).

Time-scale

The treatment with doxorubicin for 48 hours demonstrates the increase in the cell viability in Twist/FOXC2/Snail overexpressed HMLE compared to control HMLE (Saxena et al., 2011).

The inhibition of Twist or Zeb1 with small interference RNA (siRNA) induced the inhibition of cell viability compared to control MDAMB231 cells treated with doxorubicin for 48 hours (Saxena et al., 2011).

Known modulating factors

ABC transporters that are related to drug resistance are overexpressed in the EMT-activated cells (Saxena et al., 2011). The expression of PD-L1, which binds to the PD-1 on the cytotoxic T cells, is up-regulated in EMT-activated cells, which results in the inhibition of cancer immunity and the resistance to cancer therapy (Shibue & Weinberg, 2017).

Known Feedforward/Feedback loops influencing this KER

The investigation of EMT-CSC relations is important to understand the relationship between EMT and cancer malignancy. Non-CSCs in cancer can spontaneously undergo EMT and dedifferentiate into new CSC, subsequently induce the regeneration of tumorigenic potential (Marjanovic, Weinberg, & Chaffer, 2013; Shibue & Weinberg, 2017).

The plastic CSC theory demonstrates the bidirectional conversions between non-CSCs and CSCs, which may contribute to the acquisition of cancer malignancy in EMT-activated cells (Marjanovic et al., 2013).

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