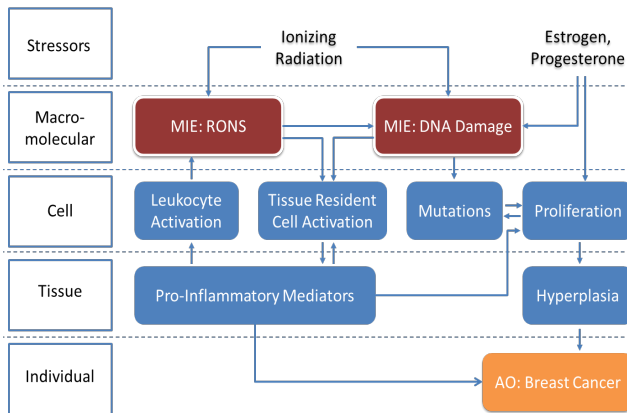


AOP 294: Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer

Short Title: RONS leading to breast cancer

Graphical Representation

AOPs for RONS and DNA damage leading to Breast Cancer



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Status

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Abstract

Knowledge about established breast carcinogens can support improved 21st century toxicological testing methods by identifying key mechanistic events. Ionizing radiation (IR) increases the risk of breast cancer, especially for women and for exposure at younger ages. We used the Adverse Outcome Pathway (AOP) framework to outline and evaluate the evidence linking ionizing radiation with breast cancer from molecular initiating events (MIE) to the adverse outcome (AO) through intermediate key events (KE). We identified prospective key events using recent literature on ionizing radiation and carcinogenesis, focusing on review articles. We searched PubMed for each key event and ionizing radiation, and used references cited in the resulting papers and targeted searches with related key words to identify additional papers. We manually curated publications and evaluated data quality. The AOP specifies that ionizing radiation directly and indirectly causes DNA damage and increases production of reactive oxygen and nitrogen species (RONS), and these are designated as MIEs. RONS lead to DNA damage (MIE) which leads to mutations (KE). Proliferation (KE) amplifies the effects of DNA damage and mutations leading to the AO of breast cancer. Separately, RONS (and DNA damage) also increase inflammation (KE). Inflammation contributes to direct and indirect effects (effects in cells not directly reached by IR) via positive feedback to RONS and DNA damage, and separately increases proliferation and the AO through pro-carcinogenic effects on cells and tissue. These MIEs and KEs overlap at multiple points with events characteristic of "background" induction of breast carcinogenesis, including hormone-responsive proliferation, oxidative activity, and DNA damage. These overlaps make the breast particularly susceptible to ionizing radiation and reinforce the importance of these MIEs and KEs as part of toxicological panels for carcinogenicity. The AOP identifies areas for additional research, including better description of the time and dose-dependence of MIEs and KEs in mammary tissues directly and indirectly exposed to IR.

This AOP extends the characteristics of mammary carcinogens beyond DNA damage, highlighting the important role in breast cancer of chemicals that increase RONS, cell proliferation, and inflammation. Chemicals that increase these biological processes should be considered potential breast carcinogens, and predictive methods should be developed to identify chemicals that increase these processes. Ultimately, this AOP will improve methods that predict chemical breast carcinogens so that exposure can be reduced.

Background

Breast cancer imposes a significant burden on women worldwide and is an important target for prevention. It is the most common invasive cancer in women with the highest rates found in North America and Europe (Ervik, Lam et al. 2016), and incidence is increasing globally (Forouzanfar, Foreman et al. 2011). In the US, the National Cancer Institute estimates that the total number of new breast cancers will increase from 283,000 to 441,000 between 2011 and 2030 (Rosenberg, Barker et al. 2015). Twin studies suggest that heritable factors explain at most a third of breast cancers and around 60% of all cancers are related to avoidable factors (Ronckers, Erdmann et al. 2005; Colditz and Wei 2012; Moller, Mucci et al. 2016), leaving significant room for prevention efforts focused on environmental factors to reduce new cases. Well-documented risk factors include tobacco and alcohol use as well as obesity, physical activity, and exposure to carcinogens (Colditz and Wei 2012).

Breast cancer incidence and risk varies with age, and hormonal and reproductive factors. Incidence increases with age, with rates among women increasing rapidly after age 30 and peaking around 75 years of age (NCI SEER 2016). Incidence is strongly influenced by the reproductive hormones estrogen and progesterone and by childbirth, which influence the proliferation and number of cells in the breast (Gertig, Stillman et al. 1999; Ronckers, Erdmann et al. 2005; Bijwaard, Brenner et al. 2010; Dall, Risbridger et al. 2017). Breast cancer risk increases with earlier puberty or later menopause (CGHFBC 2012; Bodicoat, Schoemaker et al. 2014), factors that increase cumulative estrogen and progesterone exposure and the number of proliferative menstrual cycles in the breast. Conversely, risk decreases in women with ovariectomies (Olson, Sellers et al. 2004; Press, Sullivan-Halley et al. 2011) and with menopause (CGHFBC 2012). Risk also decreases with number of pregnancies, breastfeeding, and increasing time since childbirth. This decrease in risk is thought to be related to the differentiation of stem cells in the breast during pregnancy and lactation and the decline in epithelial cell number after childbirth (Gertig, Stillman et al. 1999; Dall, Risbridger et al. 2017). Breast cancer incidence in men is less than 1% that of women, a difference attributed to low levels of estrogen and progesterone and few breast epithelial cells (Stang and Thomssen 2008).

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Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
	MIE	1632	Increase in reactive oxygen and nitrogen species (RONS) (https://aopwiki.org/events/1632)	Increase in RONS
	KE	1182	Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/events/1182)	Increase, Cell Proliferation (Epithelial Cells)
	KE	1492	Tissue resident cell activation (https://aopwiki.org/events/1492)	Tissue resident cell activation
	KE	1493	Increased Pro-inflammatory mediators (https://aopwiki.org/events/1493)	Increased pro-inflammatory mediators
	KE	1494	Leukocyte recruitment/activation (https://aopwiki.org/events/1494)	Leukocyte recruitment/activation
	AO	1194	Increase, DNA damage (https://aopwiki.org/events/1194)	Increase, DNA Damage
	AO	185	Increase, Mutations (https://aopwiki.org/events/185)	Increase, Mutations
	AO	1192	Increased, Ductal Hyperplasia (https://aopwiki.org/events/1192)	Increased, Ductal Hyperplasia
	AO	1193	N/A, Breast Cancer (https://aopwiki.org/events/1193)	N/A, Breast Cancer

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase in reactive oxygen and nitrogen species (RONS) (https://aopwiki.org/relationships/1904)	adjacent	Increase, DNA damage	High	Not Specified
Increase, DNA damage (https://aopwiki.org/relationships/1897)	adjacent	Increase, Mutations	High	Not Specified
Increase, Mutations (https://aopwiki.org/relationships/1896)	adjacent	Increase, Cell Proliferation (Epithelial Cells)	Moderate	Not Specified
Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/relationships/1907)	adjacent	Increase, Mutations	High	Not Specified
Increase, Cell Proliferation (Epithelial Cells) (https://aopwiki.org/relationships/1247)	adjacent	Increased, Ductal Hyperplasia	Not Specified	Not Specified
Increased, Ductal Hyperplasia (https://aopwiki.org/relationships/1252)	adjacent	N/A, Breast Cancer	High	Not Specified
Increase in reactive oxygen and nitrogen species (RONS) (https://aopwiki.org/relationships/1906)	adjacent	Tissue resident cell activation	Moderate	Not Specified
Increase, DNA damage (https://aopwiki.org/relationships/1898)	adjacent	Tissue resident cell activation	Moderate	Not Specified
Tissue resident cell activation (https://aopwiki.org/relationships/1763)	adjacent	Increased Pro-inflammatory mediators	Moderate	Not Specified
Increased Pro-inflammatory mediators (https://aopwiki.org/relationships/1777)	adjacent	Leukocyte recruitment/activation	Moderate	Not Specified
Leukocyte recruitment/activation (https://aopwiki.org/relationships/1905)	adjacent	Increase in reactive oxygen and nitrogen species (RONS)	High	Not Specified
Increased Pro-inflammatory mediators (https://aopwiki.org/relationships/1903)	adjacent	Increase in reactive oxygen and nitrogen species (RONS)	High	Not Specified
Increased Pro-inflammatory mediators (https://aopwiki.org/relationships/1908)	adjacent	Increase, Cell Proliferation (Epithelial Cells)	Moderate	Not Specified
Increased Pro-inflammatory mediators (https://aopwiki.org/relationships/1902)	adjacent	N/A, Breast Cancer	Moderate	Not Specified

Stressors

Name	Evidence
Ionizing Radiation	High
Other DNA damaging agents	Moderate

Ionizing Radiation

Human

Exposure to ionizing radiation is a well-established risk factor for breast cancer in people. Ionizing radiation increases the risk of death from breast cancer and other solid cancers, particularly bladder and renal cancers, as well as leukemia and other blood cancers (Ozasa, Shimizu et al. 2012). Much of the evidence for breast cancer following radiation in humans comes from therapeutic or diagnostic (typically low LET) radiation and from the atomic bombs in Japan, which released a radiation mixture featuring low LET gamma but also neutron radiation (Preston, Mattsson et al. 2002). Epidemiologic studies of women exposed to the atomic bomb in Japan (Little and McElvenny 2017), to therapeutic radiation for benign disorders (Eidemuller, Holmberg et al. 2015), childhood cancer (Henderson, Amsterdam et al. 2010; Moskowitz, Chou et al. 2014), or contralateral breast cancer (Neta, Anderson et al. 2012), or to frequent chest X-rays including TB fluoroscopy (Ma, Hill et al. 2008; Bijwaard, Brenner et al. 2010) all show a significant increase of breast cancer risk with radiation exposure.

Rodent

Rodents can be used to study mammary gland carcinogenesis in response to ionizing radiation, but formation of mammary tumors in rodents in response to ionizing radiation varies by species and by strain. Mammary tumors are common in rats (Russo 2015) and ionizing radiation increases the incidence of mammary tumors, although sensitivity to radiation varies by strain (Imaoka, Nishimura et al. 2009). Mammary tumors are rare in mice (Wagner 2004), leading to the use of genetically sensitive strains and tumor promoting viruses to study mammary tumors in mice (Wagner 2004; Russo 2015). The BALB/c mouse has a higher baseline rate of mammary tumors, and in this strain ionizing radiation increases the incidence of mammary tumors (Imaoka, Nishimura et al. 2009; Rivina, Davoren et al. 2016).

Modifying factors

Age

Women exposed to therapeutic doses of ionizing radiation at younger ages are more susceptible to breast cancer from ionizing radiation than women exposed later in life (Miller, Howe et al. 1989; Boice, Preston et al. 1991; Ma, Hill et al. 2008; Stovall, Smith et al. 2008; Berrington de Gonzalez, Curtis et al. 2010). Studies of atomic bomb survivors also show higher risk of breast cancer with decreasing age at the time of the bombing, although different models result in different conclusions about whether age at exposure acts additively (Land, Tokunaga et al. 2003) or multiplicatively (Preston, Ron et al. 2007) with regard to other breast cancer risk factors.

The stage of development at ionizing radiation exposure is also important in animals. Risk appears to be highest for IR exposures between one and seven weeks during mammary gland development and puberty (Imaoka, Nishimura et al. 2011; Imaoka, Nishimura et al. 2013; Imaoka, Nishimura et al. 2017) with lower rates in embryonic, adult (Imaoka, Nishimura et al. 2011; Imaoka, Nishimura et al. 2013), and post-estrous rats (Bartstra, Bentvelzen et al. 1998). As in humans, studies of pre-pubertal risk in animals may be affected by the impact of whole body radiation on ovaries, leading to decreased circulating reproductive hormones (Imaoka, Nishimura et al. 2011).

The effect of age is thought to be related to developmental changes occurring in the breast with puberty and with childbirth and breast feeding. The growth and development of the epithelial portion of the breast that will eventually produce and deliver milk is limited until the onset of puberty (Sternlicht, Sunnarborg et al. 2005). Undifferentiated stem cells proliferate at puberty and expand into the stroma to form branched structures and terminal ducts (Hinc and Silberstein 2005; Sternlicht, Sunnarborg et al. 2005). Stem cells are thought to be more capable of forming tumors because their long lifespan makes it more likely that they will sustain multiple mutagenic hits, frequent mitosis increases the likelihood of mutation, and because they are capable of passing any mutations on to multiple progeny which can then acquire further mutations (Imaoka, Nishimura et al. 2009; Russo 2015). Thus puberty brings an expansion in the number of vulnerable cells. Development continues to a lesser degree after puberty with each menstrual cycle.

Pregnancy or parity is protective against breast cancer from radiation. Early age of pregnancy acted multiplicatively to reduce risk from the atomic bomb (Land, Hayakawa et al. 1994), and women who have never gone through childbirth (and the associated breast differentiation) before radiation exposure have an increased risk of contralateral breast cancer from ionizing radiation while no significant increase is seen among parous women (Brooks, Boice et al. 2012). This decrease in risk of IR exposure with parity is consistent with breast cancer risk in the general population- risk of (ER+) breast cancer is higher in older women who have never had a child and lower for women who have had one or more children (after an initial increase around childbirth) (Britt, Ashworth et al. 2007; Ma, Henderson et al. 2010; Dall, Risbridger et al. 2017).

The rodent literature on IR does not offer a clear parallel to the epidemiological data, with animal exposures occurring only during or shortly after pregnancy. Rats are more sensitive to mammary cancer following IR during or shortly after pregnancy compared with virgin mice. Several studies find that cancer incidence is higher in animals exposed to ionizing radiation while pregnant and lactating (Inano, Suzuki et al. 1991; Suzuki, Ishii-Ohba et al. 1994; Inano, Suzuki et al. 1996). Post-lactational extracellular matrix also supports the metastasis of transplanted tumors (McDaniel, Rumer et al. 2006), although an early study did not report a difference in tumor incidence between virgin, pregnant, lactating, and post-lactational rats exposed to IR (Holtzman, Stone et al. 1982). However, parity is protective against spontaneous and carcinogen-induced mammary tumors in rodents (Britt, Ashworth et al. 2007; Rajkumar, Kittrell et al. 2007; Dall, Risbridger et al. 2017).

The protective effect of parity observed in humans and in spontaneous and carcinogen-induced mammary tumors is again attributed to the development and differentiation of susceptible stem cells in the breast. Proliferation increases dramatically during pregnancy before a major terminal differentiation leading to lactation (Oakes, Hilton et al. 2006; Anderson, Rudolph et al. 2007). This process is coupled with a decline in hormone sensing epithelial cells and stem cells in the mammary gland (Dall, Risbridger et al. 2017). Conversely, this pregnancy-related decrease in hormone sensing and stem cells does not apply to first pregnancies at older ages and may explain the lack of protection afforded by first parity in older women (Dall, Risbridger et al. 2017). This unique developmental timeline of the breast results in increased susceptibility to carcinogens during the proliferative phases followed by a long-term decrease in susceptibility after early pregnancy and later in life. This theory underlies the current efforts to prevent breast cancers by induction of terminal differentiation (mimicking pregnancy) in teenagers (Santucci-Pereira, George et al. 2013).

Estrogen

The modification of breast cancer risk from IR with age is likely related to the age and parity-dependent changes in hormones and their effects on the proliferation and differentiation of epithelial cells in the breast. As with spontaneous breast cancer, hormones increase the risk of breast cancer following ionizing radiation in women. Breast cancer rates following exposure to therapeutic doses of radiation (for cancers including Hodgkin's lymphoma) are lower in women who subsequently undergo premature menopause or whose treatment involved higher doses of radiation to the ovaries causing effects similar to early menopause (Travis, Hill et al. 2003; De Bruin, Spardans et al. 2009; Inskip, Robison et al. 2009; Moskowitz, Chou et al. 2014). Genetic variation in estrogen signaling also affects risk. Polymorphisms in estrogen synthesis and metabolism genes modify the risk of breast cancer after occupational or diagnostic exposure to X-rays (Sigurdson, Bhatti et al. 2009). Similarly, risk increases with and may be partially mediated by increased serum estrogen in postmenopausal atomic bomb survivors (Grant, Cologne et al. 2018).

Similarly, exposure to estrogen or the synthetic estrogen diethylstilbestrol (DES) is associated with more tumors (particularly adenocarcinomas) in rats following IR. This effect can be observed in intact rats supplemented with DES or estradiol (E2) before, concurrent with or after IR (Segaloff and Maxfield 1971; Shellabarger, Stone et al. 1976; Holtzman, Stone et al. 1979; Holtzman, Stone et al. 1981; Solleveld, van Zwieten et al. 1986; Broerse, Hennen et al. 1987; Inano, Suzuki et al. 1991). This increased effect of radiation in the presence of estrogen can also be observed in male rats treated with DES (Inano, Suzuki et al. 1996) and ovariectomized rats (OVX) treated before or after puberty with estradiol (Inano, Yamanouchi et al. 1995; Yamanouchi, Ishii-Ohba et al. 1995). Conversely, OVX (Cronkite, Shellabarger et al. 1960; Clifton, Yasukawa-Barnes et al. 1985; Solleveld, van Zwieten et al. 1986) and the anti-estrogen tamoxifen (Welsch, Goodrich-Smith et al. 1981; Lemon, Kumar et al. 1989; Peterson, Servinsky et al. 2005) reduce tumors from IR. In addition, one study reports that IR can increase circulating estrogen in rodents (Suman, Johnson et al. 2012). While this effect would be consistent with reports in postmenopausal women after the atomic bomb, the finding has not been repeated.

The effect of progesterone on carcinogenesis depends on the developmental state of the mammary gland. Progesterone does not appear to have a strong effect in pre-pubertal or immature mammary gland, which has not proliferated in response to estrogen (Inano, Yamanouchi et al. 1995). In contrast, progesterone was associated with elevated risk of carcinogenesis after IR in post-pubertal rats (Yamanouchi, Ishii-Ohba et al. 1995; Takabatake, Daino et al. 2018), consistent with a combined effect of estrogen and progesterone on breast cancer risk seen in the Women's Health Initiative trials (Chlebowski, Aragaki et al. 2015). Curiously, in some studies progesterone reduces the effect of E2 on IR-induced tumorigenesis (Inano, Yamanouchi et al. 1995; Yamanouchi, Ishii-Ohba et al. 1995). In this case the combined E2 and progesterone treatment may have actually matured the breast in a manner akin to pregnancy – estrogen levels were higher than typical for pregnancy and lactation in rats and the resulting glands were highly developed and had no terminal end buds (Inano, Yamanouchi et al. 1995; Yamanouchi, Ishii-Ohba et al. 1995).

Several studies suggest that therapeutic (Huang, Newman et al. 2000; Castiglioni, Terenziani et al. 2007; Dores, Anderson et al. 2010; Neta, Anderson et al. 2012; Horst, Hancock et al. 2014; Alkner, Ehinger et al. 2015) and environmental (VoPham, DuPre et al. 2017) ionizing radiation particularly increase the risk of estrogen receptor negative (ER-) breast tumors in women, possibly by acting on ER- stem cells in the breast. It should be noted, however, that most of these studies are in women with a history of prior cancer. On the other hand, in a study of low dose diagnostic radiation exposure and another small study of atomic exposed women there was no association between exposure and tumors' estrogen receptor status (Ma, Hill et al. 2008; Miura, Nakashima et al. 2008).

In animals, tumors formed after IR in the absence of estrogen (ovariectomized animals) are often ER- while those formed in the presence of estrogen or DES are often ER+ (Inano, Yamanouchi et al. 1995) and those formed in the presence of estrogen and progesterone are almost always ER+ (Inano, Yamanouchi et al. 1995; Yamanouchi, Ishii-Ohba et al. 1995).

Genetic susceptibility

Susceptibility to breast cancer from ionizing radiation varies with genetic background. Women with certain genetic polymorphisms in DNA damage response genes such as BRCA or ATM are more susceptible to breast cancer from ionizing radiation (Millikan, Player et al. 2005; Broeks, Braaf et al. 2007; Brooks, Teraoka et al. 2012; Bernstein, Thomas et al. 2013), particularly when exposed at a younger age (Andrieu, Easton et al. 2006; Broeks, Braaf et al. 2007; Bernstein, Haile et al. 2010; Pijpe, Andrieu et al. 2012). Polymorphisms in estrogen synthesis and metabolism also affect risk of breast cancer from IR (Sigurdson, Bhatti et al. 2009). An early study of women exposed to the atomic bomb also suggested that a surge in rapid onset cancers arose from genetically susceptible populations (Land, Tokunaga et al. 1993).

In rodents, certain strains (genetically different families of a species) are more susceptible to mammary cancer following ionizing radiation than others, indicating genetic influences on susceptibility (Shellabarger 1972; Vogel and Turner 1982; Imaoka, Nishimura et al. 2007; Rivina, Davoren et al. 2016).

Dose dependence

Breast cancer risk increases linearly across a wide range of ionizing radiation doses in humans (Miller, Howe et al. 1989; Boice, Preston et al. 1991; Preston, Mattsson et al. 2002; Preston, Ron et al. 2007; Ronckers, Doody et al. 2008; Inskip, Robison et al. 2009; Adams, Dozier et al. 2010; Eidemuller, Holmberg et al. 2015; Little and McElvenny 2017; Shore, Beck et al. 2018) and in animals (Gragtmans, Myers et al. 1984; Imaoka, Nishimura et al. 2007), although some flattening may occur at the highest doses (attributed to cell killing effects) (Imaoka, Nishimura et al. 2007; Ibrahim, Abouelkhair et al. 2012; Moskowitz, Chou et al. 2014). Cancer data at doses lower than 0.1-0.2 Gy is scarce and conflicting, with some studies showing significant increases in cancers among individuals exposed to lower doses compared with unexposed people (Preston, Mattsson et al. 2002; Sigurdson, Bhatti et al. 2009; Adams, Dozier et al. 2010), including among genetically susceptible BRCA carriers (Pijpe, Andrieu et al. 2012), while others have not (Jacrot, Mouriquand et al. 1979; Imaoka, Nishimura et al. 2007; Sasaki, Tachibana et al. 2014). While gamma radiation elicits a linear low-dose response, mammary tumor risk after higher LET radiation exhibited steeper dose-dependence at lower doses (Imaoka, Nishimura et al. 2007). Despite the higher uncertainty around individual breast cancer studies after low doses of IR, a recent review of the dose-response of solid tumors IR including several focused on breast cancer concluded that the evidence supported a linear no-threshold dose response model even at lower doses (Shore, Beck et al. 2018).

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Other DNA damaging agents

Breast carcinogenesis from IR and DNA damaging agents has more similarities than differences (Imaoka, Nishimura et al. 2009). Both IR and other DNA damaging agents form adenocarcinomas in rodents with similar pathology and gene expression, although IR also creates a much larger fraction of fibroadenomas than DNA damaging chemicals (Imaoka, Nishimura et al. 2009). Carcinogenicity for IR and chemical mammary carcinogens NMU and DMBA varies with age and exposure to ovarian hormones (Medina 2007; Imaoka, Nishimura et al. 2009; Russo 2015). Breast carcinogenesis from IR and chemical carcinogens depends strongly on developmental or ongoing exposure to ovarian hormones (Nandi, Guzman et al. 1995; Russo 2015), and estrogen status of tumors increases with ovarian hormone exposure in rats (Nandi, Guzman et al. 1995; Imaoka, Nishimura et al. 2009). The mammary gland is especially susceptible to both IR and mammary carcinogens DMBA and NMU around puberty. This is presumably because puberty is when undifferentiated cells are both large in number and will undergo major subsequent proliferative expansion, although additional factors including metabolism and expression of DNA damage repair genes contribute to variations in the age of maximal susceptibility between agents (Medina 2007; Imaoka, Nishimura et al. 2009; Imaoka, Nishimura et al. 2011; Imaoka, Nishimura et al. 2013). Consistent with general accepted risk assessment assumptions of additivity in carcinogenesis, IR has an additive effect in combination with NMU (Imaoka, Nishimura et al. 2014). Some differences between mammary carcinogens appear around the protective role of breast maturation: pregnancy appears to be more protective in rats exposed to chemical carcinogens than in rats exposed to IR.

The role of DNA damage, mutation, and proliferation outlined in this AOP would presumably apply to other DNA damaging agents while the role of RONS and inflammation is more likely to vary between DNA damaging and other agents based on their ability to induce these key events. DNA damaging agents differ in the degree, type and reparability of the DNA damage they cause. Mammary carcinogens NMU, DMBA, PhIP, and urethane mostly cause adducts with single nucleotide substitutions (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation 2006; Imaoka, Nishimura et al. 2009; Westcott, Halliwill et al. 2014; Nik-Zainal, Kucab et al. 2015; Sherborne, Davidson et al. 2015). Like ionizing radiation, mammary carcinogen PhIP can cause amplifications and NMU can cause genomic instability (Goepfert, Moreno-Smith et al. 2007; Imaoka, Nishimura et al. 2009). While IR also induces adducts, it characteristically generates complex damage and double-strand breaks leading to deletions and inversions as well as amplification and genomic instability (Pazhanisamy, Li et al. 2011; Datta, Suman et al. 2012; Mukherjee, Coates et al. 2012; Snijders, Marchetti et al. 2012; Yang, Killian et al. 2015; Behjati, Gundem et al. 2016; Mavragani, Nikitaki et al. 2017). The prevalence of complex damage and double strand breaks is likely due to the density of damage delivered by ionizing radiation, but is also attributable to oxidative activity, since IR creates an oxidative state and H₂O₂ and other oxidizing agents can also cause (less) complex damage, double strand breaks and mutations (Seager, Shah et al. 2012; Sharma, Collins et al. 2016; Cadet, Davies et al. 2017). Radiomimetic compounds (used in chemotherapy) also cause double-strand breaks and simple complex damage. Agents like bleomycin cause double strand breaks through oxidized lesions (Regulus, Duroux et al. 2007), while agents like etoposide and cisplatin cause double strand breaks by interfering with DNA replication forks (Kawashima, Yamaguchi et al. 2017).

Evidence suggests that proliferation and inflammation are also implicated in chemical carcinogenicity. The aforementioned pubertal susceptibility implies a dependence on proliferation, as does the fact that tumorigenesis following NMU depends on proliferation during treatment (Medina 2007). Like IR, NMU and DMBA promote hyperplasia in terminal end buds and ducts and ductal carcinoma in situ leading to carcinogenesis (Goepfert, Moreno-Smith et al. 2007; Medina 2007; Imaoka, Nishimura et al. 2009; Russo 2015). In terms of inflammation, some chemical carcinogens appear to share with IR an increase in inflammatory reactions in mammary stroma and a tumor-promoting effect of stroma (Russo and Russo 1996; Barcellos-Hoff and Ravani 2000; Maffini, Soto et al. 2004; Nguyen, Oketch-Rabah et al. 2011) and although bleomycin has not been characterized for its effects on mammary stroma or mammary carcinogenesis it causes lung fibrosis (an anti-inflammatory reaction) so consistently that it is used as a research model for that endpoint (Moeller, Ask et al. 2008).

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Overall Assessment of the AOP

See Annex 1 for the assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

See Appendix 2 (KEs and KERs) for the evidence supporting each key event and key event relationship.

Domain of Applicability

While the key events described here are likely relevant to all tissues after exposure to IR, it is particularly relevant to the female mammary gland. While ionizing radiation causes many kinds of cancers including leukemia, lung, bladder, and thyroid cancers (BEIR 2006; Preston, Ron et al. 2007), breast cancers are among the cancers most increased by exposure to ionizing radiation (Preston, Ron et al. 2007).

The lengthy and hormone-dependent developmental trajectory of the mammary gland is likely to be a major factor in its susceptibility to breast cancer. Numerous epidemiological and laboratory studies support the requirement for ovarian hormones in the risk of breast cancer from ionizing radiation (Grant, Cologne et al. 2018). Although at first examination breast cancer from ionizing radiation and hormones involve very different processes, in fact the hormone-dependent and ionizing radiation pathways of carcinogenesis intersect at multiple points that are part of breast development leaving the hormone-exposed breast more vulnerable to radiation. Two studies in humans and rats also suggest that IR can increase long term concentrations of circulating estrogen which would further amplify any additive effects, although additional evidence is needed (Suman, Johnson et al. 2012; Grant, Cologne et al. 2018).

One major mechanism promoting breast cancer from ionizing radiation is the proliferation of breast stem cells. Stem cells are considered to be important to initiation because of their long life and capacity to pass on mutations to many progeny. Breast tissue is responsive to estrogen and progesterone, reproductive hormones that rise at puberty and stimulate cellular proliferation with each reproductive cycle and in pregnancy. These hormonal proliferative cycles increase the risk of cancer in breast tissue (Briskin, Hess et al. 2015). IR increases the long term proliferation of stem cells in pubertal but not adult mammary gland (Nguyen, Oketch-Rabah et al. 2011; Datta, Hyde et al. 2012; Snijders, Marchetti et al. 2012; Suman, Johnson et al. 2012; Tang, Fernandez-Garcia et al. 2014). Replication of stem cells in the IR-exposed breast is therefore particularly elevated during puberty, likely contributing to the increased susceptibility to breast cancer from IR at this age.

Another vulnerability of the breast to IR is a byproduct of proliferation: mutations. Replication itself increases the likelihood of mutations, which add to mutations arising from IR and increase the likelihood of oncogenic transformation (Atashgaran, Wrin et al. 2016). Furthermore, the high replication rate of mammary gland epithelial cells during puberty and pregnancy increases reliance on homologous recombination pathways (Kass, Lim et al. 2016). Disruption of these HR processes by IR-induced mutation or increased demand for repair can increase mutation rates and increase tumorigenesis (Mahdi, Huo et al. 2018). This disruption is particularly relevant for mammary stem cells which are

highly replicating and dependent on HR but shift to NHEJ to respond to DNA damage from IR (Chang, Zhang et al. 2015). The consequence of mutations in stem cells is significant, since these cells can clonally expand to generate many mutated progeny. However, errors in stem cell division may not be the sole or primary factor driving cancer from radiation, since excess cancer risk for solid cancers at different sites from the atomic bomb are not clearly related to the number of stem cell divisions at that site (Tomasetti, Li et al. 2017).

The elevated estrogen associated with development and the estrous cycle may also have direct effects that further complement the carcinogenic effects of IR. Estrogen directly increases oxidative stress in virgin (but not parous) mice (Yuan, Dietrich et al. 2016), interferes with DNA repair (Pedram, Razandi et al. 2009; Li, Chen et al. 2014) increases mutations (Mailander, Meza et al. 2006), and increases TGF- β (Jerry, Dunphy et al. 2010). Each of these effects would increase the impact of the same events arising from IR alone.

Inflammation from the estrous cycle may also contribute to tumorigenesis following IR. Cytokines and macrophages play an integral role in mammary gland development and ductal elaboration, with alternating inflammatory, immune surveillance, and phagocytic activity occurring over each estrous cycle (Hodson, Chua et al. 2013; Atashgaran, Wrin et al. 2016; Brady, Chuntova et al. 2016). This inflammation could potentially increase IR-induced DNA damage and mutations and promote tumorigenic and invasive characteristics.

The enhancement of IR induced tumorigenesis by the estrous cycle may be replicated or further enhanced by exogenous endocrine disrupting chemicals. Indeed, evidence suggests that BPA (and presumably other estrogenic chemicals) exposure in utero can increase the mammary gland's response to progesterone during puberty (Briskin, Hess et al. 2015). This enhancement would presumably also increase the risk of breast cancer from ionizing radiation, since that risk increases with estrogen exposure and the number of menstrual cycles.

Uncertainty arising from extrapolating from rodent and human in vitro studies to human biology

Uncertainty in this pathway arises from inconsistencies in carcinogenesis between rodent and mouse species and strains and from incomplete information about the same mechanisms operating in humans. This raises questions about whether all evidence should be weighted equally.

Almost half of the data included here is from in vitro experiments on human primary or cultured cells, which should have a high degree of relevance for this pathway in humans. However, most of the human cells are not from mammary gland, and most of the mammary gland derived cells are cancer or immortalized cells that will not respond in exactly the same way as primary cells. Even this human data should therefore be interpreted with some caution.

Most of the remaining data in this AOP is from mice, with a relatively small number of rat studies. As a breast cancer model, mice share important characteristics with humans (Medina 2007; Imaoka, Nishimura et al. 2009). Mice and humans share similar epithelial cell types (Lim, Wu et al. 2010) and a similar developmental regime with the bulk of epithelial development occurring postnatally and accelerating during puberty, with differentiation during pregnancy (Medina 2007). Tumors in humans originate in the terminal ductal lobular unit, a structure that includes the lobule with secretory alveoli and the start of the collecting duct. The developmental terminal end bud structure is thought to be particularly vulnerable to carcinogens because of the presence of stem cells and proliferation, although it is not the only possible site of initiation. Similarly, tumors in mice originate in predominantly in alveoli as well as terminal end buds and small ducts (Medina 2007). Humans are more susceptible to carcinogens around puberty, and pregnancy is protective. Evidence on the role of development and reproduction in mammary carcinogenesis in mice is limited compared with rats but is consistent with sensitivity to radiation around puberty (Imaoka, Nishimura et al. 2009), and parity is protective for chemical carcinogens (Medina 2007). In addition, proliferation contributes to carcinogenesis in both mice and humans (Medina 2007).

However, mice differ from humans in some notable ways (Medina 2007). Mammary tumors are not common in mice, so susceptible strains or tumor-promoting viruses are used to increase spontaneous incidence and response to carcinogenic stimuli. This difference may be partially attributable to hormone responsiveness of tumors. Although tumors in mice depend on hormones for development, breast cancers in rats and humans are frequently hormone receptor positive, while mammary tumors in mice are not (Nandi, Guzman et al. 1995; Medina 2007; Imaoka, Nishimura et al. 2009).

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Essentiality of the Key Events

IR appears to be a "complete" carcinogen in the mammary gland in that the toxin acts as an initiator through the formation of oxidative stress and pro-mutagenic DNA damage and (the MIEs) and as a promoter through increasing inflammation and proliferation, similar to many chemical carcinogens (Russo and Russo 1996). We have high confidence in the evidence linking stressor (IR) with adverse outcome (breast cancer). The weight of evidence for the first pathway from RONS and DNA damage to Mutation and Proliferation is High while the weight of evidence for the second pathway from RONS to Inflammation to Proliferation and Breast Cancer is Moderate. These evaluations are based on the supporting evidence for all KEs and the considerations in Annex 1, and based on the need for additional evidence in the essentiality of Inflammation for the genesis of breast cancer.

This AOP could not address the large number of related topics that interact with the key events described here. These topics include events following IR that may interact with these key events such as immune surveillance (which may change with the inflammatory environment after IR (Schreiber, Old et al. 2011; Barcellos-Hoff 2013; Lumniczky and Safrany 2015); IR effect on survival/apoptosis and interactions of apoptosis with inflammation, mutation, compensatory proliferation, and selection process; changes to DNA repair; and the role of epigenetics in carcinogenesis from IR (Daino, Nishimura et al. 2018). This AOP also does not address other influences on these key events beyond reproductive hormones and typical breast development. Subsequent contributions to this AOP should elaborate on these points.

	Defining question	High (Strong)	Moderate	Low (Weak)
2. Support for essentiality of KEs	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
MIE: Increase in reactive oxygen and nitrogen species (RONS)	Essentiality is High. The most significant support comes from the relatively large number of studies using antioxidants or other interventions to reduce RONS, which show a reduction in DNA damage and mutations. Additional support comes from experiments increasing external oxidants like H2O2, which show that RONS are independently capable of causing DNA damage and mutations. Uncertainties arise from the smaller effects of RONS on DNA damage compared with ionizing radiation. Mammary gland relevance is less certain due to the relatively few experiments in breast tissue.			

KE/AO: Increase in DNA damage	Essentiality is High. The essentiality of this MIE to cancer is generally accepted. Supporting evidence comes from application of mutagenic agents: the increase in DNA damage precedes mutations, proliferation, and tumorigenesis. Further indirect evidence comes from evidence for MIE1, in which antioxidants that reduce DNA damage also reduce mutations and chromosomal damage. Finally, mutations in DNA repair genes increase the risk of tumors.
KE/AO: Increase in mutation	Essentiality is High. The contribution of this MIE to cancer is generally accepted. Evidence comes from knock-out and knock-in experiments, which find that mutations in certain key genes increase tumorigenesis. However, an ongoing debate pits the singular importance of mutations against a significant role for the tissue microenvironment. This debate is fueled by transplant studies that show the importance of tissue environment for tumorigenesis and suggesting that mutations may not be sufficient for tumorigenesis.
KE: Increase, Cell Proliferation (epithelial cells)	Essentiality is High. Cellular proliferation is a key characteristic of cancer cells and can lead to hyperplasia, an intermediate phase in the development of tumorigenesis. Proliferation also increases the number of cells with mutations, which can further promote proliferation and/or changes to the local microenvironment.
KE/AO: Increase, Ductal Hyperplasia	Essentiality is High. Evidence comes from transplant experiments showing that non-proliferating tissue is less tumorigenic than proliferating lesions, and from interventions that reduce both proliferation and tumors. Further evidence comes from animals that are resistant to both mammary gland proliferation and tumors from ionizing radiation. Uncertainty arises from conflicting evidence on the tumorigenicity of hyperplasia, the absence of hyperplasia observed before some tumors, and spontaneous regression of tumors.
KEs: Tissue Resident Cell Activation, Increased Pro-inflammatory mediators, Leukocyte recruitment/activation	Essentiality is Moderate. These key events were reviewed as a group. Evidence comes from using genetic modifications, antibodies, and antioxidants to reduce inflammatory and anti-inflammatory factors. These interventions reduce DNA damage, mutations, and mechanisms contributing to tumorigenesis and invasion. Uncertainty arises from conflicting effects in different genetic backgrounds and in different organs.

MIE1: Increase in RONS

Essentiality is High. The most significant support comes from the relatively large number of studies using antioxidants or other interventions to reduce RONS, which show a reduction in DNA damage and mutations. Additional support comes from experiments increasing external oxidants like H₂O₂, which show that RONS are independently capable of causing DNA damage and mutations. Uncertainties arise from the smaller effects of RONS on DNA damage compared with ionizing radiation. Mammary gland relevance is less certain due to the relatively few experiments in breast tissue.

Multiple studies support the hypothesis that elevated RONS is a key part of the adverse outcome pathway for breast cancer from ionizing radiation. The strongest evidence comes from studies showing that reducing RONS also reduces DNA damage in irradiated cells and bystander cells, including genomic instability observed at later time points after IR. Free radical and NADPH oxidase inhibitors reduce the effect of IR on DNA nucleotide damage, double strand breaks, chromosomal damage, and mutations in isolated DNA and cultured cells (Winyard, Faux et al. 1992; Douki, Ravanat et al. 2006; Choi, Kang et al. 2007; Jones, Riggs et al. 2007; Ameziane-El-Hassani, Boufraqech et al. 2010; Ameziane-El-Hassani, Talbot et al. 2015; Manna, Das et al. 2015) and on nucleotide damage and double strand breaks in vivo (Pazhanisamy, Li et al. 2011; Ozyurt, Cevik et al. 2014). RONS reduction after ionizing radiation also reduces genomic instability in animals and in cloned cell lines (Dayal, Martin et al. 2008; Dayal, Martin et al. 2009; Pazhanisamy, Li et al. 2011; Bensimon, Biard et al. 2016). RONS are similarly implicated in IR effects in bystander cells. Antioxidants (including a nitric oxide scavenger) and oxidase inhibitors added before or after radiation reduce micronuclei and gamma-H2AX formation in bystander cells (Azzam, De Toledo et al. 2002; Yang, Asaad et al. 2005; Yang, Anzenberg et al. 2007). Antioxidant activity also reduces the inflammatory response to IR in animals and cultured skin cells (Berruyer, Martin et al. 2004; Das, Manna et al. 2014; Ozyurt, Cevik et al. 2014; Haddadi, Rezaeyan et al. 2017; Zhang, Zhu et al. 2017).

RONS are sufficient to trigger subsequent key events in this AOP. Extracellularly applied or intracellularly generated ROS (which also facilitates the formation of RNS) are capable of creating DNA damage in vitro including base damage, single and double strand breaks, and chromosomal damage (Oya, Yamamoto et al. 1986; Dahm-Daphi, Sass et al. 2000; Nakamura, Purvis et al. 2003; Gradzka and Iwanenko 2005; Ismail, Nystrom et al. 2005; Driessens, Versteheyte et al. 2009; Berdelle, Nikolova et al. 2011; Lorat, Brunner et al. 2015; Stanicka, Russell et al. 2015) and mutations (Sandhu and Birnboim 1997; Ameziane-El-Hassani, Boufraqech et al. 2010; Seager, Shah et al. 2012; Sharma, Collins et al. 2016). Similarly, decreased antioxidant activity and higher RONS is observed in cells with genomic instability (Dayal, Martin et al. 2008; Buonanno, de Toledo et al. 2011). To our knowledge, no experiments have tested whether elevating intracellular RONS alone in one group of cells can cause bystander effects in another.

Evidence in Mammary Gland

The increase of RONS following IR has been shown in a wide range of cells, in vivo and in vitro, including epithelial cells, and in two studies in mammary epithelial cells (Jones, Riggs et al. 2007; Bensimon, Biard et al. 2016). Both mammary cell studies also show increased RONS and DNA damage over a day after IR in vitro and link DNA damage with elevated RONS.

Uncertainties or Inconsistencies

The mitigating effects of antioxidants on IR-generated DNA damage support the essentiality of RONS in producing DNA damage and mutations. However, externally applied RONS is less effective than IR at generating double strand breaks and mutations (Sandhu and Birnboim 1997; Dahm-Daphi, Sass et al. 2000; Gradzka and Iwanenko 2005; Ismail, Nystrom et al. 2005). One possible explanation for this discrepancy is that IR may elicit a higher concentration of localized RONS than can be achieved with external application of H₂O₂. IR deposits energy and oxidizes molecules within a relatively small area over a rapid timescale potentially permitting a very high local concentration which could precede or overwhelm local buffering capacity. In contrast, extracellularly applied H₂O₂ would interact with many antioxidants and other molecules on its way to the nucleus, where the concentration would slowly reach a lower steady state.

As expected for RONS as a key event for DNA damage from IR, DNA damage from IR and H₂O₂ are additive in cells (Dahm-Daphi, Sass et al. 2000; Driessens, Versteheyte et al. 2009). Unexpectedly however, inhibiting glutathione (which should increase or sustain the effects of RONS), increases DNA damage from H₂O₂ but not IR. This lack of effect of glutathione inhibition on IR conflicts with multiple studies showing decreased DNA damage from IR with anti-oxidants. One possible explanation is that the concentration or reaction rate of glutathione is already inadequate to buffer the elevated RONS from IR, so further inhibition has no measurable effect.

KE/AO: Increase in DNA damage

Essentiality is High. The essentiality of this MIE to cancer is generally accepted. Supporting evidence comes from application of mutagenic agents: the increase in DNA damage precedes mutations, proliferation, and tumorigenesis. Further indirect evidence comes from evidence for MIE1, in which antioxidants that reduce DNA damage also reduce mutations and chromosomal damage. Finally, mutations in DNA repair genes increase the risk of tumors.

Increases or decreases in DNA damage are associated with corresponding increases or decreases in downstream key events in the pathway to breast cancer. An external agent (ionizing radiation) that increases DNA damage (Padula, Ponzinibbio et al. 2016) also causes chromosomal damage and increased mutations (Sandhu and Birnboim 1997; Jones, Riggs et al. 2007; Denissova, Nasello et al. 2012; Fibach and Rachmilewitz 2015), transforms cells (Yang, Craise et al. 1992; Yang, Georgy et al. 1997; Unger, Wienberg et al. 2010), and causes tumors (Poirier and Beland 1994; Little 2009). Polymorphisms or mutations in DNA repair genes affect tumor formation after ionizing radiation in animals (Yu, Okayasu et al. 2001; Umehiko, Fujisawa et al. 2005) and in people (Millikan, Payer et al. 2005; Andrieu, Easton et al. 2006; Broeks, Braaf et al. 2007; Bernstein, Haile et al. 2010; Brooks, Teraoka et al. 2012; Pijpe, Andrieu et al. 2012; Bernstein, Thomas et al. 2013). Consistent with these findings, antioxidants that reduce DNA damage from stressors like IR also reduce chromosomal aberrations and micronuclei arising from those stressors (Azzam, De Toledo et al. 2002; Choi, Kang et al. 2007; Jones, Riggs et al. 2007).

Evidence in mammary gland

The majority of research on the effects of IR on DNA damage has been performed in tissues other than mammary gland, but several studies suggest that effects in the mammary gland (and its consequences) would be consistent with other tissues. Oxidative DNA damage in mammary cells increases immediately after exposure to IR (Haegel, Wolfe et al. 1998), and double stranded breaks, micronuclei, and (later) chromosomal aberrations appear two hours to six days after IR exposure in vivo and in vitro (Soler, Pampalona et al. 2009; Snijders, Marchetti et al. 2012; Hernandez, Terradas et al. 2013). Genomic instability was reported in genetically susceptible cells after a month of higher doses of IR (4 doses of 1.8 Gy but not 0.75 Gy) (Snijders, Marchetti et al. 2012).

KE/AO: Increase in mutation

Essentiality is High. The contribution of this MIE to cancer is generally accepted. Evidence comes from knock-out and knock-in experiments, which find that mutations in certain key genes increase tumorigenesis. However, an ongoing debate pits the singular importance of mutations against a significant role for the tissue microenvironment. This debate is fueled by transplant studies that show the importance of tissue environment for tumorigenesis and suggesting that mutations may not be sufficient for tumorigenesis.

Mutations increase transformation in culture (Wang, Su et al. 2011) and proliferation and tumors in mice (Radice, Ferreira-Cornwell et al. 1997; Umehiko, Fujisawa et al. 2005; de Ostrovich, Lambertz et al. 2008; Podsypanina, Politi et al. 2008; Francis, Bergsied et al. 2009; Gustin, Karakas et al. 2009; Francis, Chakrabarti et al. 2011; Tao, Xiang et al. 2017). Restoring function in mutated genes regresses tumors in animals (Martins, Brown-Swigart et al. 2006; Podsypanina, Politi et al. 2008). Mutations are common in tumors (Haag, Hsu et al. 1996; Greenman, Stephens et al. 2007; Stratton, Campbell et al. 2009; CGAN (Cancer Genome Atlas Network) 2012; Vandin, Upfal et al. 2012; Garraway and Lander 2013; Vogelstein, Papadopoulos et al. 2013; Yang, Killian et al. 2015) and tumors are largely clonal, suggesting that individual mutations offer the tumor evolutionary advantages (Wang, Waters et al. 2014; Yates, Gerstung et al. 2015; Begg, Ostrovnyaya et al. 2016).

Evidence in mammary gland

Many of the studies in support of the proliferative and tumorigenic role of mutations are in mammary gland or breast cancers. Further support for including DNA damage and mutation in the mechanistic pathway linking ionizing radiation with breast cancer comes from the observation that variants in DNA repair genes increase the risk of mammary tumors in animals after IR (Yu, Okayasu et al. 2001; Umesako, Fujisawa et al. 2005) and increase breast cancer after IR (Millikan, Player et al. 2005; Andrieu, Easton et al. 2006; Broeks, Braaf et al. 2007; Bernstein, Haile et al. 2010; Brooks, Teraoka et al. 2012; Pijs, Andrieu et al. 2012; Bernstein, Thomas et al. 2013). BRCA is perhaps the best known DNA repair gene linked with breast cancer risk, and several studies of these studies have suggested a link between BRCA mutation status and increased susceptibility to breast cancer following ionizing radiation, particularly in women exposed at younger ages (Pijs, Andrieu et al. 2012).

Uncertainties or Inconsistencies

Mutations alone are not sufficient or even essential for tumor growth in mammary glands. Mammary tumor incidence following ionizing radiation varies significantly by sex and depends on the presence of ovarian hormones (Cronkite, Shellabarger et al. 1960; Segaloff and Maxfield 1971; Shellabarger, Stone et al. 1976; Holtzman, Stone et al. 1979; Holtzman, Stone et al. 1981; Welsch, Goodrich-Smith et al. 1981; Clifton, Yasukawa-Barnes et al. 1985; Solleveld, van Zwieten et al. 1986; Broerse, Hennen et al. 1987; Lemon, Kumar et al. 1989; Inano, Suzuki et al. 1991; Inano, Suzuki et al. 1996; Peterson, Servinsky et al. 2005). Tumor growth from transplanted tumor cells varies with age, parity, and lactational status (Maffini, Calabro et al. 2005; McDaniel, Rumer et al. 2006), and stroma treated with carcinogens or IR supports tumors from pre-malignant epithelial cells (Barcellos-Hoff and Ravani 2000; Maffini, Soto et al. 2004; Nguyen, Oketch-Rabah et al. 2011). While the mechanisms underlying these contextual factors have not been clearly identified, the proliferative effect of hormones on the mammary gland may serve to amplify damaged and mutated cells and modify the stromal environment to increase the likelihood of cellular transformation. Inflammatory responses including the release of cytokines and the activation of inflammatory and anti-inflammatory signaling pathways likely also amplify the effects of DNA damage and mutations through many of the same mechanisms.

KE: Increase in proliferation

Essentiality is High. Cellular proliferation is a key characteristic of cancer cells (Hanahan and Weinberg 2011) and can lead to hyperplasia, an intermediate phase in the development of tumorigenesis. Proliferation also increases the number of cells with mutations, which can further promote proliferation and/or changes to the local microenvironment.

Evidence in mammary gland

Multiple studies show that mammary gland proliferates after IR or chemical carcinogen treatment prior to the appearance of mammary tumors. Epithelial cells proliferate following IR in vitro (Mukhopadhyay, Costes et al. 2010) and in vivo (Nguyen, Oketch-Rabah et al. 2011; Sniijders, Marchetti et al. 2012; Suman, Johnson et al. 2012; Tang, Fernandez-Garcia et al. 2014). Increasing proliferation leads to hyperplasia (Korkaya, Paulson et al. 2009). Proliferative nodules and hyperplasia appear in mammary terminal end bud, alveolae, and ducts of rats and mice after exposure to chemical carcinogens (Beuving, Bern et al. 1967; Beuving, Faulkin et al. 1967; Russo, Saby et al. 1977; Purnell 1980) and ionizing radiation (Faulkin, Shellabarger et al. 1967; Ullrich and Preston 1991; Imaoka, Nishimura et al. 2006). Proliferating foci precede the development of tumors (Haslam and Bern 1977; Purnell 1980) and form tumors more effectively than non-proliferating tissue (Deome, Faulkin et al. 1959; Beuving 1968; Rivera, Hill et al. 1981).

Supporting the essentiality of these proliferative processes to tumorigenesis, ACI rats that exhibit no mammary proliferation or hyperplasia following IR are resistant to tumors following IR (Kutanzi, Koturbash et al. 2010). Interventions reducing proliferation in susceptible PyVT and BALB/c mice also reduce mammary tumors (Luo, Fan et al. 2009; Connelly, Barham et al. 2011; Tang, Fernandez-Garcia et al. 2014).

Uncertainties or Inconsistencies

Some studies report carcinogenesis in the absence of hyperplasia (Sinha and Dao 1974) and others do not find increased tumorigenesis from transplanted hyperplasia (Beuving, Bern et al. 1967; Haslam and Bern 1977; Sinha and Dao 1977). The failure of some proliferative foci to form tumors and the regression of some tumors when formed (Haslam and Bern 1977; Purnell 1980; Korkola and Archer 1999) suggests that proliferation may not be sufficient for sustained tumorigenesis in mammary gland.

KE/AO: Increase, ductal hyperplasia

Essentiality is High. Evidence comes from transplant experiments showing that non-proliferating tissue is less tumorigenic than proliferating lesions, and from interventions that reduce both proliferation and tumors. Further evidence comes from animals that are resistant to both mammary gland proliferation and tumors from ionizing radiation. Uncertainty arises from conflicting evidence on the tumorigenicity of hyperplasia, the absence of hyperplasia observed before some tumors, and spontaneous regression of tumors.

Hyperplasia signals the presence of excess proliferation (a key characteristic of cancer cells (Hanahan and Weinberg 2011)) and represents an intermediate phase in the development of tumorigenesis.

Evidence in mammary gland

Multiple studies show that mammary gland proliferates after IR or chemical carcinogen treatment prior to the appearance of mammary tumors. Proliferative nodules and hyperplasia appear in mammary terminal end bud, alveolae, and ducts of rats and mice after exposure to chemical carcinogens (Beuving, Bern et al. 1967; Beuving, Faulkin et al. 1967; Russo, Saby et al. 1977; Purnell 1980) and ionizing radiation (Faulkin, Shellabarger et al. 1967; Ullrich and Preston 1991; Imaoka, Nishimura et al. 2006). Proliferating foci precede the development of tumors (Haslam and Bern 1977; Purnell 1980) and form tumors more effectively than non-proliferating tissue (Deome, Faulkin et al. 1959; Beuving 1968; Rivera, Hill et al. 1981). Adenocarcinomas in rats appear to preferentially form from terminal end bud hyperplasia (Haslam and Bern 1977; Russo, Saby et al. 1977; Purnell 1980), similar to the origin of many breast cancers for humans and for some mice after IR (Medina and Thompson 2000).

Supporting the essentiality of these proliferative processes to tumorigenesis, ACI rats that exhibit no mammary proliferation or hyperplasia following IR are resistant to tumors following IR (Kutanzi, Koturbash et al. 2010). Interventions reducing proliferation in susceptible PyVT and BALB/c mice also reduce mammary tumors (Luo, Fan et al. 2009; Connelly, Barham et al. 2011).

Uncertainties or Inconsistencies

Some studies report carcinogenesis in the absence of hyperplasia (Sinha and Dao 1974) and others do not find increased tumorigenesis from transplanted hyperplasia (Beuving, Bern et al. 1967; Haslam and Bern 1977; Sinha and Dao 1977). The failure of some lesions to form tumors and the regression of some tumors when formed (Haslam and Bern 1977; Purnell 1980; Korkola and Archer 1999) suggests that hyperplasia alone may not be sufficient for sustained tumorigenesis in mammary gland.

KEs: Tissue resident cell activation, Increase, Pro-inflammatory mediators, Leukocyte Recruitment/Activation

Essentiality is Moderate. These key events were reviewed as a group. Evidence comes from using genetic modifications, antibodies, and antioxidants to reduce inflammatory and anti-inflammatory factors. These interventions reduce DNA damage, mutations, and mechanisms contributing to tumorigenesis and invasion. Uncertainty arises from conflicting effects in different genetic backgrounds and in different organs.

Tumors and tumor cells exhibit features of inflammation, and inflammation is generally understood to promote transformation and tumor progression by supporting multiple hallmarks of cancer including oxidative activity and DNA damage, survival and proliferation, angiogenesis, and invasion and metastasis (Iliopoulos, Hirsch et al. 2009; Hanahan and Weinberg 2011; Esquivel-Velazquez, Ostoa-Saloma et al. 2015).

Many of these cancer promoting effects of inflammation can be seen following exposure to ionizing radiation (Bisht, Bradbury et al. 2003; Elahi, Suraweera et al. 2009; Nguyen, Oketch-Rabah et al. 2011; Bouchard, Bouvette et al. 2013; Nguyen, Fredlund et al. 2013; Ila-Bochaca, Ouyang et al. 2014). Inflammatory pathways are commonly activated in breast and mammary cancers following IR (Nguyen, Oketch-Rabah et al. 2011; Nguyen, Fredlund et al. 2013; Ila-Bochaca, Ouyang et al. 2014). Polymorphisms in inflammation genes are associated with breast cancer risk from IR in radiation technologists (Schonfeld, Bhatti et al. 2010) and with susceptibility to intestinal adenoma following IR in mice (Elahi, Suraweera et al. 2009). Cytokines TGF- β and IL6 transform primary human mammospheres and pre-malignant mammary epithelial cell lines in vitro and make them tumorigenic in vivo (Sansone, Storci et al. 2007; Iliopoulos, Hirsch et al. 2009; Nguyen, Oketch-Rabah et al. 2011), and inflammation related factors COX2 and TGF- β are required for the full effect of IR on DNA damage and transformation in vitro and mammary tumor growth and invasion in vivo (Bisht, Bradbury et al. 2003; Nguyen, Oketch-Rabah et al. 2011).

One mechanism of cancer promotion involves oxidative activity and DNA damage: inflammation in response to IR increases oxidative activity in a positive feedback loop leading to increased DNA lesions and mutations. Oxidative activity mediates the increase in inflammatory markers (TNF- α and neutrophil markers) in bladder and kidney (Ozyurt, Cevik et al. 2014), and TNF- α and neutrophils increase oxidative activity (Jackson, Gajewski et al. 1989; Stevens, Bucurenci et al. 1992; Zhang, Zhu et al. 2017). Inflammatory activity from neutrophils and TNF- α and NF- κ B-dependent COX2 and NO damage DNA and increase mutations by increasing oxidative activity (Jackson, Gajewski et al. 1989; Zhou, Ivanov et al. 2005). The mutations can be reduced by blocking the inflammatory factors NF- κ B, COX2, TNF- α , or nitric oxide, or with antioxidants (Jackson, Gajewski et al. 1989; Zhou, Ivanov et al. 2005; Zhou, Ivanov et al. 2008; Zhang, Zhu et al. 2017). Antibodies to TNF- α or TGF- β reduce DNA damage in bone marrow (Burr, Robinson et al. 2010; Rastogi, Coates et al. 2012) and CHO cells (Han, Chen et al. 2010). Inhibiting TNF- α also reduces genomic instability in directly irradiated (but not bystander) lymphocytes (Moore, Marsden et al. 2005) and in bone marrow of CBA/Ca mice susceptible to IR-induced leukemia but not resistant C57BL/6 mice (Lorimore, Mukherjee et al. 2011). Inhibiting inflammatory factors NF- κ B or iNOS reduces IR-induced bystander mutations in lung fibroblasts (Zhou, Ivanov et al. 2008).

Inflammatory pathways activated by IR are also capable of promoting tumor growth and metastasis. Exposure to IR or RONS sensitizes mammary epithelial cells to respond to TGF- β - which is widely activated by IR (Ehrhart, Segarini et al. 1997). IR and TGF- β signaling leads to an epithelial to mesenchymal (EMT)-like transition, which disrupts the expression and distribution of cell adhesion molecules and multicellular organization and promotes invasion (Park, Henshall-Powell et al. 2003; Andarawewa, Erickson et al. 2007; Andarawewa, Costes et al. 2011; Iizuka, Sasatani et al. 2017). This mechanism resembles wound healing (Koh and DiPietro 2011; Perez, Vago et al. 2014; Landen, Li et al. 2016), but also resembles malignancy - invasive breast cancer cell lines overexpress TGF- β and respond to TGF- β with increased invasion (Kim, Kim et al. 2004; Gomes, Terra et al. 2012).

The response to TGF- β likely involves an increase in senescence in fibroblasts. IR-induced senescence releases a suite of signaling molecules including pro-inflammatory IL6 and proteases (MMPs) (Tsai, Chuang et al. 2005; Liakou, Mavrogenatou et al. 2016; Perrott, Wiley et al. 2017). The signaling molecules released by IR-senescent fibroblasts promote the disorganized tissue structure of mammary epithelial cells and the growth, EMT, and invasion of breast cancer epithelial cells or mutant epithelial cells (Tsai, Chuang et al. 2005; Liakou, Mavrogenatou et al. 2016; Perrott, Wiley et al. 2017) and 3D mammary tumor models (Sourisseau, Harrington et al. 2011). The induction of senescence in fibroblasts by IR requires TGF- β (Liakou, Mavrogenatou et al. 2016), and the release of the pro-invasive signaling molecules involves an IL-1 dependent activation of NF- κ B (Perrott, Wiley et al. 2017). Senescence following IR also selects for a post-senescent variant of epithelial cell that is more conducive to tumorigenesis (Mukhopadhyay, Costes et al. 2010).

IL6 may play an important function in the carcinogenic response to IR. IL6 is expressed in mouse mammary gland after IR (Bouchard, Bouvette et al. 2013). IL6 is produced by IR-senescent fibroblasts, but may also be expressed by epithelial cells after IR since primary human mammospheres and pre-malignant mammary epithelial cell lines respond to IL6 with increased IL6 expression (Sansone, Storci et al. 2007; Iliopoulos, Hirsch et al. 2009). IL6 promotes the mobility and tumorigenesis of normal and breast cancer epithelial cells (Sansone, Storci et al. 2007; Sasser, Sullivan et al. 2007; Studebaker, Storci et al. 2008; Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010). This activity depends on transcription factor NOTCH3, which supports the renewal of stem-like cell populations (Sansone, Storci et al. 2007), and NOTCH has been implicated in multiple other studies in the proliferative response to IR in mammary epithelia (Nguyen, Oketch-Rabah et al. 2011; Marusyk, Tabasum et al. 2014; Tang, Fernandez-Garcia et al. 2014). The NF- κ B/IL6/STAT3 signaling pathway generates cancer stem cells in multiple types of breast cancer cells (Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Iliopoulos, Hirsch et al. 2011) and is also implicated in colon and other cancers (Iliopoulos, Jaeger et al. 2010). The inflammation related transcription factor NF- κ B also contributes to mammary tumorigenesis and metastasis in PyVT mice, in which mammary tumors are induced by expression of an MMTV-driven oncogene (Connelly, Barham et al. 2011). Interestingly, breast cancer fibroblasts and fibroblasts from common sites of breast cancer metastasis (bone, lung) express IL6. IL6 is required for the growth and tumor promoting effects of these fibroblasts on ER-positive cancer cells in vitro and in vivo. ER-negative breast epithelial cells release autocrine IL6 and may therefore be less dependent on IL6 from fibroblasts, although IL6 also transforms these cells (Sasser, Sullivan et al. 2007; Studebaker, Storci et al. 2008; Iliopoulos, Hirsch et al. 2009).

Inflammation is suspected to play a role in the indirect effects of radiation, in which cells not directly targeted by radiation exhibit effects including DNA damage and RONS (Lorimore and Wright 2003; Mukherjee, Coates et al. 2014; Sprung, Ivshkevich et al. 2015). In addition to the IR-induced release of inflammatory signals that are diffusible and can trigger systemic immune responses, inflammatory factors COX2 and TGF-β are produced in bystander cells that are not directly irradiated but are exposed to irradiated cells or media (Zhou, Ivanov et al. 2005; Zhou, Ivanov et al. 2008; Chai, Calaf et al. 2013; Chai, Lam et al. 2013; Wang, Wu et al. 2015).

Inflammatory factors TGF-β, TNF-α, COX2, and NO are implicated in the RONS (Shao, Folkard et al. 2008; Zhou, Ivanov et al. 2008; Wang, Wu et al. 2015). DNA damage (Dickey, Baird et al. 2009; Han, Chen et al. 2010; Dickey, Baird et al. 2012; Chai, Calaf et al. 2013; Chai, Lam et al. 2013; Wang, Wu et al. 2015) and mutations (Zhou, Ivanov et al. 2005; Zhou, Ivanov et al. 2008) observed in bystander cells and in the appearance of genomic instability (Moore, Marsden et al. 2005; Natarajan, Gibbons et al. 2007; Lorimore, Chrystal et al. 2008; Lorimore, Mukherjee et al. 2011) after IR. Further evidence for inflammation in indirect effects of IR come from tumors arising from mammary epithelial cells transplanted into IR exposed cleared fat pads: inflammation-related genes and pathways are upregulated or enriched in the gene expression patterns of these indirectly IR-induced tumors (Nguyen, Oketch-Rabah et al. 2011; Nguyen, Fredlund et al. 2013; Illa-Bochaca, Ouyang et al. 2014).

Evidence in mammary gland

Many of the studies above that link inflammatory signals with increased oxidative activity, senescence, EMT, bystander effects, genomic instability, and tumorigenesis, and metastasis use mammary tissue. Since inflammation-related signals are reported after IR in mammary gland (Barcellos-Hoff, Derynck et al. 1994; Dickey, Baird et al. 2009; Datta, Hyduke et al. 2012; Snijders, Marchetti et al. 2012; Bouchard, Bouvette et al. 2013; Wang, Wu et al. 2015) inflammation likely contributes to many of the effects of IR in this tissue.

Uncertainties or inconsistencies

The effects of inflammation can be both pro and anti-tumorigenic. For example, in addition to TGF-β's role in EMT, in mammary epithelial cells TGF-β is essential to apoptosis of DNA damaged cells including damage following ionizing radiation (Ewan, Henshall-Powell et al. 2002), thus limiting genomic instability (Maxwell, Fleisch et al. 2008). Inflammatory factors TNF-α and COX2 play a similar role in bone marrow of C57BL/6 mice (Lorimore, Rastogi et al. 2013). By eliminating cells with severe DNA damage and curtailing genomic instability, apoptosis (and therefore TGF-β or TNF-α) limits the appearance of major (possibly carcinogenic) mutations following ionizing radiation. However, apoptosis (and thus TGF-β or TNF-α) can indirectly promote tumorigenesis through compensatory proliferation (Loree, Koturbash et al. 2006; Fogarty and Bergmann 2017).

Genetic background also influences the interaction between inflammation and tumorigenesis. Polymorphisms in inflammatory genes influence susceptibility to intestinal cancer following IR (Elahi, Suraweera et al. 2009). In the SPRET outbred mouse higher baseline TGF-β during development decreases tumor incidence following lower doses of IR (0.1 Gy), possibly by reducing ductal branching and susceptibility (Zhang, Lo et al. 2015). Conversely, the BALB/c mouse susceptible to mammary tumors after IR has a lower baseline TGF-β (and a polymorphism in a DNA damage repair-related gene). Early (4 hours) after low dose (0.075 Gy) IR BALB/c mice have suppressed immune pathways and macrophage response but increased IL6, COX2, and TGF-β pathway activation in mammary gland compared to the tumor-resistant C57BL/6 mouse (Snijders, Marchetti et al. 2012; Bouchard, Bouvette et al. 2013). By 1 week after IR, the BALB/c mice show TGF-β dependent inflammation in the mammary gland, and by 1 month after IR, their mammary glands show proliferation (Nguyen, Martinez-Ruiz et al. 2011; Snijders, Marchetti et al. 2012), suggesting that TGF-β is associated with inflammation, proliferation, and mammary tumorigenesis in these mice. Consistent with this pattern, BALB/c mice that are heterozygous for TGF-β are more resistant to mammary tumorigenesis following IR (Nguyen, Oketch-Rabah et al. 2011). However, the BALB/c mouse also has a polymorphism in a DNA repair gene associated with IR-induced genomic instability (Yu, Okayasu et al. 2001), making it difficult to distinguish potentially overlapping mechanisms.

While inflammatory signals are associated with bystander effects including DNA damage, genomic instability, and mutation, these effects vary between organs in vivo (Chai, Calaf et al. 2013; Chai, Lam et al. 2013), by genotype (Coates, Rundle et al. 2008; Lorimore, Chrystal et al. 2008; Lorimore, Mukherjee et al. 2011), and by cell type (Chai, Calaf et al. 2013). Further research will be required to identify all the underlying factors determining differences in bystander effects, but one variable is the appearance of a protective apoptotic response to cytokines under some conditions (Lorimore, Mukherjee et al. 2011; Lorimore, Rastogi et al. 2013).

One major piece of conflicting evidence comes from a direct test of the essentiality of inflammation to IR-induced carcinogenesis. In a mouse model of lymphoma, a mutation preventing the PIDD/NEMO dependent activation of NF-κB blocks early IR-induced activation of NF-κB (4-24 h) and production of TNF-α (5-48 h) but not lymphoma, suggesting that activation of these inflammatory factors is not essential in this time period (Bock, Krumschnabel et al. 2013). However, this study examined only day one post-IR time points for NF-κB activity, and did not block production of IL6. Later activation of NF-κB or activation of other inflammation-related factors including IL6 and TGF-β could therefore potentially have contributed to lymphoma.

Weight of Evidence Summary

Support for biological plausibility of KERs

	Defining question	High (Strong)	Moderate	Low (Weak)
	a. Is there a mechanistic relationship between KEup and KEdown consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs, but the structural or functional relationship between them is not understood.
MIE1 => MIE2 <i>Increase in RONS leads to increase in DNA damage</i>	High. Reactive oxygen and nitrogen species from oxygen and respiratory activity are generally acknowledged to damage DNA under a range of cellular conditions.			
MIE2 => KE1 <i>DNA damage leads to mutations</i>	High. DNA damage in the form of nucleotide damage, single strand and double strand breaks, and complex damage can generate mutations, particularly when a damaged cell undergoes replication.			
KE1 => KE2 <i>Mutations can promote proliferation</i>	High. Multiple mechanisms limit the proliferation of cells in normal biological systems. Mutations in many of the genes controlling these mechanisms promote proliferation.			
KE2 => KE1 <i>Proliferation leads to mutation</i>	High. Proliferation is generally acknowledged to increase mutations through incorporating or amplifying the impact of unrepaired DNA damage as mutations.			
KE2 => AO <i>Proliferation promotes breast cancer and invasion</i>	High. It is generally accepted that proliferation contributes to cancer. Proliferation increases the number of cells with mutations, which can further promote proliferation and/or changes to the local microenvironment.			
MIE1 => KE3 <i>Increase in RONS leads to inflammation</i>	Moderate. Damage from RONS can activate some inflammatory and anti-inflammatory pathways (TLR, TGF-β), and RONS are an essential part of the primary signaling pathways of multiple inflammatory and anti-inflammatory pathways (TLR4, TNF-α, TGF-β, NFKB).			
KE3 => MIE1 <i>Inflammation leads to an increase in RONS</i>	High. Inflammation is commonly understood to generate RONS via inflammatory signaling and activated immune cells.			
KE3 => KE2 <i>Inflammation leads to proliferation</i>	High. Inflammation is generally understood to lead to proliferation during recovery from inflammation.			
KE3 => AO <i>Inflammation promotes breast cancer and invasion</i>	Moderate. Tissue environment is known to be a major factor in carcinogenesis, and inflammatory processes are implicated in the development and invasiveness of breast and other cancers.			

Empirical support for KERs

	Defining questions	High (Strong)	Moderate	Low (Weak)
Empirical support for KERs	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KEdown and is the incidence of KEup > than that for KEdown? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data.	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don't align with hypothesized AOP
MIE1 => MIE2 <i>Increase in RONS leads to increase in DNA damage</i>	High. Multiple studies show an increase in DNA damage with RONS treatment as well as dependent changes in both RONS and DNA damage in response to stressors. DNA damage increases with RONS dose, and temporal concordance between RONS and DNA damage events following ionizing radiation is consistent with a causative relationship, although few studies examine multiple doses and time points. A small number of studies do not find double strand breaks at physiological doses, or report an increase in one key event but not the other.			

MIE2 => KE1 DNA damage leads to mutations	High. It is generally accepted that DNA damage leads to mutations. Empirical support comes in part from the observation that agents which increase DNA damage also cause mutations, that DNA damage precedes the appearance of mutations, and that interventions to reduce DNA damage also reduce mutations. None of the identified studies measure both outcomes over the same range of time points. This constitutes a readily addressable data gap.
KE1 => KE2 Mutations can promote proliferation	Moderate. Mutations that promote proliferation are frequently found in cancers, and both mutation and proliferation occur in response to tumorigenic stressors like ionizing radiation. Although not measured together after stressors, mutations appear over the same time frame or prior to the appearance of proliferation. Multiple uncertainties and conflicting evidence weaken this key event relationship. The two key events differ in their dose response- mutation but not proliferation increases with ionizing radiation dose. Furthermore, a single mutation is not necessarily sufficient to increase proliferation- proliferation typically requires multiple mutations or a change in the surrounding environment. In mammary tissue, stromal state strongly influences the proliferative nature of epithelial cells – even epithelial cells with mutated tumor suppressors may be unable to form tumors in the absence of stromal changes.
KE2 => KE1 Proliferation leads to mutation	High. We did not evaluate the empirical support for this KER in response to IR. However proliferation or mitosis is required for some types of DNA damage to be made permanent and heritable, and further DNA damage including mutation promoting double strand breaks can occur when cells divide before DNA repair is complete.
KE2 => AO Proliferation promotes breast cancer and invasion	High. Carcinogenic agents increase proliferation and hyperplasia as well as tumors. Proliferation and hyperplasia appear prior to or at the same time as tumors, grow into carcinomas, and form mammary tumors more effectively than non-proliferating tissue. Disruption of proliferation is associated with decreased tumor growth, and tumor resistant rats do not show proliferation. However, the discrepancy between the non-linear proliferative and linear mammary tumor response to carcinogen dose coupled with evidence of independent occurrences of proliferation and tumorigenesis suggests that while proliferation and hyperplasia likely promote carcinogenesis, additional factors also contribute to carcinogenesis.
MIE1 => KE3 Increase in RONS leads to inflammation	Moderate. Both RONS and inflammation increase in response to agents that increase either RONS or inflammation. Multiple studies show dose-dependent changes in both RONS and inflammation in response to stressors including ionizing radiation and antioxidants. RONS have been measured at the same or earlier time points as inflammatory markers, but additional studies are needed to characterize the inflammatory response at the earliest time points to support causation. Uncertainties come from the positive feedback from inflammation to RONS potentially interfering with attempts to establish causality, and from the large number of inflammation related factors with differing responses to stressors and experimental variation.
KE3 => MIE1 Inflammation leads to an increase in RONS	High. Signals arising from inflammation can be both pro- and anti-inflammatory, and both can have effects on RONS and downstream key events. Multiple inflammation-related factors increase RONS or oxidative damage, and ionizing radiation increases both inflammation-related signaling and RONS or oxidative damage over the same time points. Interventions to reduce inflammation also reduce RONS. The dose-dependence of the response to stressors is generally consistent between the two key events, although this is based on a small number of studies with some conflicting evidence.
KE3 => KE2 Inflammation leads to proliferation	High. We did not evaluate the empirical support for this KER in response to IR. However, inflammation is generally understood to promote proliferation and survival
KE3 => AO Inflammation promotes breast cancer and invasion	Moderate. Interventions to increase inflammatory factors increase the carcinogenic potential of targeted and non-targeted cells. Inflammation is documented at earlier time points than tumorigenesis or invasion- within minutes or hours compared to days to months for carcinogenesis, consistent with an inflammatory mechanism of tumorigenesis and invasion. Inhibition of cytokines, inflammatory signaling pathways, and downstream effectors of inflammation activity prevent transformation, tumorigenesis, and invasion following IR or stimulation of inflammatory pathways. However, the key event and the adverse outcome differ in their dose-response to ionizing radiation: inflammation always does not increase linearly with dose, while breast cancer and invasion does. Uncertainty arises from the multifunctional nature of inflammation-related pathways which may be pro- or anti-inflammatory and pro- or anti-carcinogenic based on context. Both pro- and anti-inflammatory factors may contribute to carcinogenesis- further research will be required to identify the context of each.

Considerations for Potential Applications of the AOP (optional)

Because of the long latency of mammary tumors, the two-year rodent carcinogenicity bioassay is the primary assay for the adverse outcome of breast cancer. The assay is included in the OECD Test No. 451 and 453 for carcinogenicity and combined toxicity and carcinogenicity. Mammary tumors are also reported in short term, sub-chronic, and chronic toxicity tests, but these tests are less sensitive due to their shorter duration.

This AOP is relevant to guideline tests addressing DNA damage and mutation. MIE2: Increase in DNA damage is relevant to OECD Test Nos. 473, 475, 483, 487, and 489, which detect DNA damage in the form of single and double strand breaks, chromosomal damage and micronuclei, as well as some forms of nucleotide damage. KE1: Increase in mutation is relevant to OECD Test Nos. 471, 476, 488, and 490 for in vitro and in vivo mutations. To our knowledge no guideline tests address increases in RONS, proliferation, or inflammation, although some in vitro tests in ToxCast or in development elsewhere may reflect changes in these key events.

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Appendix 1

List of MIEs in this AOP

Event: 1632: Increase in reactive oxygen and nitrogen species (RONS) (<https://aopwiki.org/events/1632>)

Short Name: Increase in RONS

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	MolecularInitiatingEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

The following stressors increase this key event: ionizing radiation.

Ionizing Radiation

Reactive oxygen and nitrogen species are created by the interaction of ionizing radiation with tissue. When ionizing radiation encounters water or extracellular or intracellular components, it releases energy. This energy ejects electrons from atoms and molecules, and the ejected electrons pass energy on to neighboring molecules. Since the majority of biological tissue is composed of water molecules, ionizing radiation results in the radiolysis of water to hydroxyl radicals, which can interact to form additional reactive molecules. This reaction is generally accepted. Because RONS have such a short half-life, their appearance has been historically measured by their effect on the cell (e.g. in terms of DNA damage), and only more recently characterized using molecular probes that directly reflect their occurrence.

The time course of RONS following ionizing radiation has been described using molecular probes- primarily the non-specific fluorescent probe for ROS DCHF as well as non-specific lipid peroxidation. ROS levels increase at multiple time points: in vitro immediately following radiation (Denissova, Nasello et al. 2012; Yoshida, Goto et al. 2012; Martin, Nakamura et al. 2014), around 15 minutes later (Narayanan, Goodwin et al. 1997; Saenko, Cieslar-Pobuda et al. 2013), hours to days (Lyng, Seymour et al. 2001; Yang, Asaad et al. 2005; Choi, Kang et al. 2007; Du, Gao et al. 2009; Das, Manna et al. 2014; Werner, Wang et al. 2014; Ameziane-El-Hassani, Talbot et al. 2015; Manna, Das et al. 2015; Zhang, Zhu et al. 2017), and in vivo intestinal epithelial cells and bone marrow stem cells showed elevated ROS up to a year after IR exposure of the animal (Pazhanisamy, Li et al. 2011; Datta, Suman et al. 2012). In intestinal epithelial cells, widespread ROS expression over a period of weeks would require transgenerational expression of ROS, indicating that a cell with increased RONS can pass that characteristic to its daughter cells.

Multiple mechanisms underlie the increase in RONS after IR. The early (15 minute) and later (days to weeks) elevation in ROS is associated with increased NADPH-oxidase production of superoxide and H₂O₂ (Narayanan, Goodwin et al. 1997; Ameziane-El-Hassani, Talbot et al. 2015), and intermediate (hours to days) and chronic ROS elevation has been associated with mitochondrial respiration (Dayal, Martin et al. 2009; Datta, Suman et al. 2012; Saenko, Cieslar-Pobuda et al. 2013). The increase in mitochondrial respiration may be supported by nitric oxide, which increases around 8 hours after IR and remains elevated through at least day 2. A chronic (1 year) ROS effect of IR was not observed in cell culture when cell divisions were limited, potentially implicating cell division in sustaining chronic RONS (Suzuki, Kashino et al. 2009). RONS can also be indirectly initiated by ionizing radiation in neighboring cells via unknown soluble factors, possibly including extracellular H₂O₂, which is elevated immediately and in the first week following IR (Driessens, Versteheyte et al. 2009; Ameziane-El-Hassani, Boufragech et al. 2010; Ameziane-El-Hassani, Talbot et al. 2015). Elevated intracellular ROS was observed in cells after exposure to media from IR-exposed cells (Narayanan, Goodwin et al. 1997; Lyng, Seymour et al. 2001; Yang, Asaad et al. 2005), and protein carbonylation and lipid oxidation reflecting RONS activity was elevated in cells 20 passages after exposure to media from IR cells (Buonanno, de Toledo et al. 2011), suggesting that the effect of IR on RONS can penetrate well beyond the directly exposed cells in both space and time.

Few studies have measured RONS at multiple doses of ionizing radiation, and the time points, doses, and cell types tested for dose response vary between studies along with the dose-dependence. Two studies report dose-dependence of RONS measured with lipid peroxidation or DCHF in response to a few doses between 0.5 and 12 Gy IR (Jones, Riggs et al. 2007; Saenko, Cieslar-Pobuda et al. 2013), dose-dependence of ROS only at lower doses below 1 Gy (Werner, Wang et al. 2014), or non-linear dose-dependence (Narayanan, Goodwin et al. 1997). Dose-dependent RONS responses are also reported in extracellular media (Driessens, Versteheyte et al. 2009), and in bystander cells not directly exposed to IR (Narayanan, Goodwin et al. 1997), even after multiple generations in culture (Buonanno, de Toledo et al. 2011). ROS appears to be more dose-dependent immediately after IR and after 24 hours following IR with less dose-dependence at times in between (Narayanan, Goodwin et al. 1997; Saenko, Cieslar-Pobuda et al. 2013; Zhang, Zhu et al. 2017), possibly reflecting different mechanisms of ROS generation. These studies use probes for ROS or indicators of oxidation, but none that we are aware of explicitly measures indicators of RNS at different doses of IR.

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Domain of Applicability

This KE is broadly applicable across species.

Key Event Description

Reactive oxygen and nitrogen species (RONS) are highly reactive oxygen- and nitrogen-based molecules that often contain or generate free radicals. Key molecules include superoxide ($[O_2]^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($[OH]^\bullet$), lipid peroxide (ROOH), nitric oxide ($[NO]^\bullet$), and peroxyxynitrite ($[ONOO]^-$) (Dickinson and Chang 2011; Egea, Fabregat et al. 2017)

RONS are generated in the course of cellular respiration, metabolism, cell signaling, and inflammation (Dickinson and Chang 2011; Egea, Fabregat et al. 2017). Superoxide and hydrogen peroxide are commonly produced by the mitochondrial electron transport chain and cytochrome c and by membrane bound NADPH oxidases and related molecules. Hydrogen peroxide is also made by the endoplasmic reticulum in the course of protein folding. Nitric oxide is produced at the highest levels by nitric oxide synthase in endothelial cells and phagocytes. The other species are produced by reactions with superoxide or peroxide, or by other free radicals or enzymes.

RONS activity is principally local. Most reactive oxygen species (ROS) have short half-lives, ranging from nano- to milliseconds, so diffusion is limited, while reactive nitrogen species (RNS) nitric oxide or peroxyxynitrite can survive long enough to diffuse across membranes (Calcerrada, Peluffo 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, Fabregat et al. 2017). The effects of ROS and RNS are countered by cellular antioxidants, with glutathione and peroxiredoxins playing a major role (Dickinson and Chang 2011).

Glutathione is slower but broad acting, while peroxiredoxins act quickly and are specific to peroxides. Peroxiredoxins are effective at low peroxide concentrations but can be deactivated at higher concentrations, suggesting the cellular response to peroxides may sometimes be non-linear.

Although their existence is limited temporally and spatially, reactive oxygen species (ROS) interact with other RONS or with other nearby molecules to produce more ROS and participate in a feedback loop to amplify the ROS signal, which can increase Reactive Nitrogen Species (RNS). Both ROS and RNS also move into neighboring cells and ROS can increase intracellular RONS signaling in neighboring cells (Egea, Fabregat et al. 2017).

RONS can modify a range of targets including amino acids, lipids, and nucleic acids to inactivate or alter target functionality (Calcerrada, Peluffo et al. 2011; Dickinson and Chang 2011; Go and Jones 2013; Ravanat, Breton et al. 2014; Egea, Fabregat et al. 2017). For example, phosphatases including the tumor suppressor PTEN can be reversibly deactivated by oxidation, and the movement of HDAC4 is peroxide dependent. Elevated ROS are implicated in proliferation and maintenance of stem cell population size (Dickinson and Chang 2011) and conversely in differentiation of stem cells and oncogene-induced senescence (Egea, Fabregat et al. 2017).

How it is Measured or Detected

RONS is typically measured using fluorescent or other probes that react with RONS to change state, or by measuring the redox state of proteins or DNA (Dickinson and Chang 2011; Wang, Fang et al. 2013; Griendling, Touyz et al. 2016). Optimal methods for RONS detection have high sensitivity, selectivity, and spatiotemporal resolution to distinguish transient and localized activity, but most methods lack one or more of these parameters.

Molecular probes that indicate the presence of RONS species vary in specificity and kinetics (Dickinson and Chang 2011; Wang, Fang et al. 2013; Griendling, Touyz et al. 2016). Small molecule fluorescent probes can be applied to any tissue in vitro, but cannot be finely targeted to different cellular compartments. The non-selective probe DCHF was widely used in the past, but can produce false positive signals and is no longer recommended. Newer more selective small molecule probes such as boronate-based molecules are being developed but are not yet widely used. Alternatively, fluorescent protein-based probes can be genetically engineered, expressed in vivo, and targeted to cellular compartments and specific cells. However, these probes are very sensitive to pH in the physiological range and must be carefully controlled. EPR (electron paramagnetic resonance spectroscopy) provide the most direct and specific detection of free radicals, but requires specialized equipment.

Alternative methods involve the detection of redox-dependent changes to cellular constituents such as proteins, DNA, lipids, or glutathione (Dickinson and Chang 2011; Wang, Fang et al. 2013; Griendling, Touyz 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.

Table 1. Common methods for detecting oxidative activity

Target	Name	Method	Strengths/Weaknesses
Hydrogen peroxide-extracellular	AmplexRed	Small molecule fluorescent probes	Can be applied to any tissue in vitro.
Hydrogen peroxide-mitochondrial	MitoPy1	Small molecule fluorescent probes	Can be applied to any tissue in vitro.
Hydrogen peroxide	HyPer	Protein-based fluorescent probes	Sensitive, can be targeted to specific cells and compartments. Slower and pH sensitive.
Hydrogen peroxide	HyPer3	Protein-based fluorescent probes	Rapid kinetics and larger dynamic range, can be targeted to specific cells and compartments. Sensitive to pH, less sensitive to H_2O_2 .
Hydrogen peroxide	Boronate-based indicators	Small molecule fluorescent probe	Selective for H_2O_2 but can interact with peroxyxynitrite.
Superoxide-intracellular	DHE (dihydroethidium)	Small molecule fluorescent probe	Can be applied to any tissue in vitro, but not targeted to different compartments.
Superoxide-intracellular	cpYFP	Protein-based fluorescent probes	Reversible. Can be targeted to specific cells and compartments.
Superoxide-mitochondrial	MitoSox	Small molecule fluorescent probe	Can be applied to any tissue in vitro.
Superoxide-mitochondrial	mt-cpYFP	Protein-based fluorescent probes	Reversible. Can be targeted to specific cells and compartments.
Superoxide-extracellular	nitroblue tetrazolium	Small molecule fluorescent probe	Can be applied to any tissue in vitro.
Superoxide-intracellular or extracellular	various trityl probes	EPR	Very specific, but requires specialized equipment, not as sensitive in tissue.
Nitric oxide	Fe[DETCl] ₂ and Fe[MGD] ₂	EPR	Very specific, but requires specialized equipment, not as sensitive in tissue.

Nitric oxide	DAF-FM	Small molecule fluorescent probe	Can be applied to any tissue in vitro, but not targeted to different compartments
Peroxynitrite	EMPO	EPR	Very specific, but requires specialized equipment, not as sensitive in tissue.
Peroxynitrite	Boronate-based indicators	Small molecule fluorescent probe	Selective for H ₂ O ₂ but can interact with (is inhibited by) peroxynitrite.
Peroxynitrite	8-nitroguanine (DNA) content	HPLC-MS/MS	Destruction of sample required for measurement.
Non-specific oxidation	DCHF	Small molecule fluorescent probe	Very non selective, and can produce false positive signals.
Non-specific oxidation	roGFP or FRET	Protein-based fluorescent probes	Slow acting. Good to look at steady state activity.
Non-specific oxidation	ratio of reduced to oxidized glutathione or cysteine	Redox state detectors	Slow acting. Good to look at steady state activity. Destruction of sample required for measurement.
Non-specific oxidation	8-oxoguanine (DNA) or protein carbonyl content	HPLC-MS/MS	Destruction of sample required for measurement.
Non-specific oxidation	TBARS (thiobarbituric acid reactive substance)	Lipid peroxidation	Destruction of sample required for measurement.

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List of Key Events in the AOP

Event: 1182: Increase, Cell Proliferation (Epithelial Cells) (<https://aopwiki.org/events/1182>)

Short Name: Increase, Cell Proliferation (Epithelial Cells)

Key Event Component

Process	Object	Action
cell proliferation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
epithelial cell

Evidence for Perturbation by Stressor

Ionizing Radiation

While higher doses of ionizing radiation cause cell death in the short term (especially of dividing cells), IR is associated with delayed proliferation in vitro and in vivo. In vitro, IR can promote the proliferation/expansion in p16-suppressed and immortal epithelial populations as well as in bystander CHO cells co-cultured with IR-exposed cells (Han, Chen et al. 2010; Mukhopadhyay, Costes et al. 2010; Tang, Fernandez-Garcia et al. 2014). In vivo, IR increases apoptosis and compensatory proliferation in adult rats (Loree, Koturbash et al. 2006), and long term expression of proliferation in adolescent but not adult mammary gland (Datta, Hyde et al. 2012; Snijders, Marchetti et al. 2012; Suman, Johnson et al. 2012), possibly via the expansion of a population of stem-like cells in vivo (Nguyen, Oketch-Rabah et al. 2011; Tang, Fernandez-Garcia et al. 2014). This proliferation appears to be associated with TGF- β /Notch activity (Tang, Fernandez-Garcia et al. 2014) and nitric oxide (Han, Chen et al. 2010). IR also increases mammary hyperplasia (Faulkin, Shellabarger et al. 1967; Imaoka, Nishimura et al. 2006). While IR can induce senescence in epithelial cells, IR selects for a post-senescent variant of epithelial cell which would be more conducive to tumorigenesis (Mukhopadhyay, Costes et al. 2010).

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Key Event Description

Proliferation occurs when changes in external signals release inhibitory controls limiting entry into the cell cycle, and oncogenic mutations act via these same pathways to generate abnormal proliferation (Hanahan and Weinberg 2011; Weber, Desai et al. 2017). Inhibitory signals such as contact inhibition or TGF-β (Polyak, Kato et al. 1994; Francis, Bergsied et al. 2009) stabilize the mechanisms limiting entry into the cell cycle. Proliferative signals such as those following progesterone or estrogen (Croce 2008; Weber, Desai et al. 2017) or compensatory proliferation after apoptosis (Fogarty and Bergmann 2017) relieve inhibition and enable cells to enter the cell cycle. Mutations that inactivate inhibitory signals (tumor suppressors) or activate proliferative signals (oncogenes) promote proliferation outside of the normal biological context (Gustin, Karakas et al. 2009; Francis, Chakrabarti et al. 2011; Hanahan and Weinberg 2011; Weber, Desai et al. 2017). Abnormal proliferation is typically met with apoptosis or senescence, so additional mutations or other mechanisms are required to escape these additional levels of control for proliferation to continue indefinitely (Garbe, Bhattacharya et al. 2009; Shay and Wright 2011; Fernald and Kurokawa 2013).

Proliferation increases mutations as DNA damage and replication errors become integrated into the genome (Kiraly, Gong et al. 2015). Proliferation can also promote the expansion of existing cells with proliferative mutations. Genomic mutations favoring further proliferation are positively selected from among the expanded cells, resulting in the accumulation of mutational errors and moving the organism further towards cancer. Different clonal populations can also collaborate to promote growth (Marusyk, Tabassum et al. 2014; Franco, Tyson et al. 2016).

How it is Measured or Detected

Past cellular proliferation can be measured directly using labels that are incorporated into cells upon cell division (BRDU or cytoplasmic proliferation dyes) or indirectly by measuring a change in population size. Ongoing current proliferation can be quantified by labeling a protein associated with the cell cycle (e.g. Ki67). Methods for measuring proliferation were reviewed in (Romar, Kupper et al. 2016) and summarized in Table 1.

Table 1. Common methods for detecting proliferation

Target	Name	Method	Strengths/Weaknesses
Past proliferation	Nucleoside analog incorporation (BRDU)	Microscopy	Stable, so can see proliferation from a specific time point onward. Can be used in vivo. BRDU must be labeled with a secondary fluorescent or other label for visualization, so it cannot be measured in living cells.
Past proliferation	Cytoplasmic proliferation dyes: carboxyfluorescein diacetate succinimidyl ester (CFSE).	Microscopy	Enables quantification of successive cell divisions and differentiation between slowly and rapidly cycling cells. Cells survive analysis, so these dyes can be used as part of ongoing experiments. The dyes are better suited to in vitro experiments.
Past proliferation	Cell counting	Microscopy	An increase in cell numbers over time could represent proliferation or a decrease in apoptosis. Better suited to in vitro experiments, unless a label can be used to uniquely label a population of cells.
Ongoing proliferation rate	Ki67 probe	Microscopy	Labels all non-G0 phase proliferating cells. Labeling requires permeabilization so examination terminates the experiment.

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Event: 1492: Tissue resident cell activation (<https://aopwiki.org/events/1492>)

Short Name: Tissue resident cell activation

Key Event Component

Process	Object	Action
cell activation involved in immune response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory (https://aopwiki.org/aops/17)	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Macaca fascicularis	Macaca fascicularis		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9541)
rat	Rattus norvegicus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
zebrafish	Danio rerio		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Extend to at least invertebrates

Not to plants and not to single-celled organisms

BRAIN:

Neuroinflammation is observed in human, monkey, rat, mouse, and zebrafish, in association with neurodegeneration or following toxicant exposure. Some references (non-exhaustive list) are given below for illustration:

In human: Vennetti et al., 2006

In monkey (Macaca fascicularis): Charleston et al., 1994, 1996

In rat: Little et al., 2012; Zurich et al., 2002; Eskes et al., 2002

In mouse: Liu et al., 2012

In zebrafish: Xu et al., 2014.

LIVER:

Human [Su et al., 2002; Kegel et al., 2015; Boltjes et al., 2014]

Rat [Luckey and Peterson, 2001]

Mouse [Dalton et al., 2009]

Key Event Description

Tissue resident cell activation is considered as a hallmark of inflammation irrespective of the tissue type. Strategically placed cells within tissues respond to noxious stimuli, thus regulating the recruitment of neutrophil and the initiation and resolution of inflammation (Kim and Luster, 2015). Examples for these cells are resident immune cells, parenchymal cells, vascular cells, stromal cells, or smooth muscle cells. These cells may be specific for a certain tissue, but they have a common tissue-independent role.

Under healthy conditions there is a homeostatic state, characterized as a generally quiescent cellular milieu. Various danger signals or alarmins that are involved in induction of inflammation like pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) activate these resident cells in affected tissues.

Examples of well-characterized DAMPs (danger signals or alarmins) (Saïd-Sadier and Ojcius, 2012)

DAMPs	Receptors	Outcome of receptor ligation
Extracellular nucleotides (ATP, ADP, adenosine)	P1, P2X and P2Y receptors (ATP, ADP); A1, A2A, A2B and A3 receptors (adenosine)	Dendritic cell (DC) maturation, chemotaxis, secretion of cytokines (IL-1 β , IL-18), inflammation
Extracellular heat shock proteins	CD14, CD91, scavenger receptors, TLR4, TLR2, CD40	DC maturation, cytokine induction, DC migration to lymph nodes
Extracellular HMGB1	RAGE, TLR2, TLR4	Chemotaxis, cytokine induction, DC activation, neutrophil recruitment, inflammation, activation of immune cells
Uric acid crystals	CD14, TLR2, TLR4	DC activation, cytokine induction, neutrophil recruitment, gout induction
Oxidative stress	Intracellular redox-sensitive proteins	Cell death, release of endogenous DAMPs, inflammation
Laminin	Integrins	Neutrophil recruitment, chemotaxis
S100 proteins or calgranulins	RAGE	Neutrophil recruitment, chemotaxis, cytokine secretion, apoptosis
Hyaluronan	TLR2, TLR4, CD44	DC maturation, cytokine production, adjuvant activity

Activation refers to a phenotypic modification of the resident cells that includes alterations in their secretions, activation of biosynthetic pathways, production of pro-inflammatory proteins and lipids, and morphological changes. While these represent a pleiotropic range of responses that can vary with the tissue, there are a number of common markers or signs of activation that are measurable.

Examples of Common markers are

- NF-κB
- AP-1
- Jnk
- P38/mapk

These described commonalities allow the use of this KE as a hub KE in the AOP network. However, despite the similarities in the inflammatory process, the type of reactive cells and the molecules triggering their reactivity may be tissue-specific. Therefore, for practical reasons, a tissue specific description of the reactive cells and of the triggering factors is necessary in order to specify in a tissue-specific manner, which cell should be considered and what should be measured.

BRAIN

The most easily detectable feature of brain inflammation or neuroinflammation is activation of microglial cells and astrocytes. It is evidenced by changes in shape, increased expression of certain antigens, and accumulation and proliferation of the glial cells in affected regions (Aschner, 1998; Graeber & Streit, 1990; Monnet-Tschudi et al., 2007; Streit et al., 1999; Kraft and Harry, 2011; Claycomb et al., 2013). Upon stimulation by cytokines, chemokines or inflammatory agents (e.g. from pathogens or from damaged neurons), both glial cell types activate inflammatory signaling pathways, which result in increased expression and/or release of inflammatory mediators such as cytokines, eicosanoids, and metalloproteinases (Dong & Benveniste, 2001) (cf KE: pro-inflammatory mediators, increased), as well as in the production of reactive oxygen species (ROS) and nitrogen species (RNS) (Brown & Bal-Price, 2003). Different types of activation states are possible for microglia and astrocytes, resulting in pro-inflammatory or anti-inflammatory signaling, and other cellular functions (such as phagocytosis) (Streit et al., 1999; Nakajima and Kohsaka, 2004). Therefore, neuroinflammation can have both neuroprotective/neuroreparative and neurodegenerative consequences (Carson et al., 2006; Monnet-Tschudi et al., 2007; Aguzzi et al., 2013; Glass et al., 2010). Under normal physiological conditions, microglial cells survey the nervous system for neuronal integrity (Nimmerjahn et al., 2005) and for invading pathogens (Aloisi, 2001; Kreutzberg, 1995; Kreutzberg, 1996; Rivest, 2009). They are the first type of cell activated (first line of defense), and can subsequently induce astrocyte activation (Falsig, 2008). Two distinct states of microglial activation have been described (Gordon, 2003; Kigerl et al., 2009; Maresz et al., 2008; Mosser & Edwards, 2008; Perego et al.; Ponomarev et al., 2005): The M1 state is classically triggered by interferon-gamma and/or other pro-inflammatory cytokines, and this state is characterized by increased expression of integrin alpha M (Itgam) and CD86, as well as the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6), and it is mostly associated with neurodegeneration. The M2 state is triggered by IL-4 and IL-13 (Maresz et al., 2008; Perego et al., 2011; Ponomarev et al., 2007) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes. The activation of astrocytes by microglia-derived cytokines or TLR agonists resembles the microglial M1 state (Falsig 2006). Although classification of the M1/M2 polarization of microglial cells may be considered as a simplification of authentic microglial reaction states (Ransohoff, 2016), a similar polarization of reactive astrocytes has been described recently Liddlelow et al., 2017): Interleukin-1 alpha (IL-1α), TNF and subcomponent q (C1q) released by activated microglial cells induce A1-reactive astrocytes, which lose the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis and induce the death of neurons and oligodendrocytes.

Regulatory examples using the KE

Measurement of GFAP in brain tissue, whose increase is a marker of astrocyte reactivity, is required by the US EPA in rodent toxicity studies for fuel additives (40 CFR 79.67). It has been used on rare occasions for other toxicant evaluations.

LIVER:

Kupffer cells (KCs) are a specialized population of macrophages that reside in the liver; they were first described by Carl Wilhelm von Kupffer (1829–1902) [Haubrich 2004]. KCs constitute 80%-90% of the tissue macrophages in the reticuloendothelial system and account for approximately 15% of the total liver cell population [Bouwens et al., 1986]. They play an important role in normal physiology and homeostasis as well as participating in the acute and chronic responses of the liver to toxic compounds. Activation of KCs results in the release of an array of inflammatory mediators, growth factors, and reactive oxygen species. This activation appears to modulate acute hepatocyte injury as well as chronic liver responses including hepatic cancer. Understanding the role KCs play in these diverse responses is key to understanding mechanisms of liver injury [Roberts et al., 2007]. Besides the release of inflammatory mediators including cytokines, chemokines, lysosomal and proteolytic enzymes KCs are a main source of TGF-β1 (transforming growth factor-beta 1, the most potent profibrogenic cytokine). In addition latent TGF-β1 can be activated by KC-secreted matrix metalloproteinase 9 (MMP-9) [Winwood and Arthur, 1993; Luckey and Peeterson, 2001] through the release of biologically active substances that promote the pathogenic process. Activated KCs also release ROS like superoxide generated by NOX (NADPH oxidase), thus contributing to oxidative stress. Oxidative stress also activates a variety of transcription factors like NF-κB, PPAR-γ leading to an increased gene expression for the production of growth factors, inflammatory cytokines and chemokines. KCs express TNF-α (Tumor Necrosis Factor-alpha), IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for hepatic stellate cells (HSCs) and induce the expression of PDGF receptors on HSCs which enhances cell proliferation. Expressed TNF-α, TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are not only pro-inflammatory active but also capable of inducing death receptor-mediated apoptosis in hepatocytes [Guo and Friedman, 2007; Friedman 2002; Roberts et al., 2007]. Under conditions of oxidative stress macrophages are further activated which leads to a more enhanced inflammatory response that again further activates KCs through cytokines (Interferon gamma (IFNγ), granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α), bacterial lipopolysaccharides, extracellular matrix proteins, and other chemical mediators [Kolios et al., 2006; Kershenovich Stalnikowitz and Weissbrod 2003].

Besides KCs, the resident hepatic macrophages, infiltrating bone marrow-derived macrophages, originating from circulating monocytes are recruited to the injured liver via chemokine signals. KCs appear essential for sensing tissue injury and initiating inflammatory responses, while infiltrating Ly-6C+ monocyte-derived macrophages are linked to chronic inflammation and fibrogenesis. The profibrotic functions of KCs (HSC activation via paracrine mechanisms) during chronic hepatic injury remain functionally relevant, even if the infiltration of additional inflammatory monocytes is blocked via pharmacological inhibition of the chemokine CCL2 [Baeck et al., 2012; Tacke and Zimmermann, 2014].

KC activation and macrophage recruitment are two separate events and both are necessary for fibrogenesis, but as they occur in parallel, they can be summarised as one KE.

Probably there is a threshold of KC activation and release above which liver damage is induced. Pre-treatment with gadolinium chloride (GdCl₃), which inhibits KC function, reduced both hepatocyte and sinusoidal epithelial cell injury, as well as decreased the numbers of macrophages appearing in hepatic lesions and inhibited TGF-β1 mRNA expression in macrophages. Experimental inhibition of KC function or depletion of KCs appeared to protect against chemical-induced liver injury [Ide et al., 2005].

How it is Measured or Detected

In General:

Measurement **targets** are cell surface and intracellular markers; the specific markers may be cell and species-specific.

Available **methods** include cytometry, immunohistochemistry, gene expression sequencing; western blotting, ELISA, and functional assays.

BRAIN

Neuroinflammation, i.e. the activation of glial cells can be measured by quantification of cellular markers (most commonly), or of released mediators (less common). As multiple activation states exist for the two main cell types involved, it is necessary to measure several markers of neuroinflammation:

1. Microglial activation can be detected based on the increased numbers of labeled microglia per volume element of brain tissue (due to increase of binding sites, proliferation, and immigration of cells) or on morphological changes. A specific microglial marker, used across different species, is CD11b. Alternatively various specific carbohydrate structures can be stained by lectins (e.g. IB4). Beyond that, various well-established antibodies are available to detect microglia in mouse tissue (F4/80), phagocytic microglia in rat tissue (ED1) or more generally microglia across species (Iba1). Transgenic mice are available with fluorescent proteins under the control of the CD11b promoter to easily quantify microglia without the need for specific stains.
2. The most frequently used astrocyte marker is glial fibrillary acidic protein, GFAP (99% of all studies) (Eng et al., 2000). This protein is highly specific for astrocytes in the brain, and antibodies are available for immunocytochemical detection. In neuroinflammatory brain regions, the stain becomes more prominent, due to an upregulation of the protein, a shape change/proliferation of the cells, and/or better accessibility of the antibody. Various histological quantification approaches can be used. Occasionally, alternative astrocytic markers, such as vimentin of the S100beta protein, have been used for astrocyte staining (Struzynska et al., 2007). Antibodies for complement component 3 (C3), the most characteristic and highly upregulated marker of A1 neurotoxic reactive astrocytes are commercially available.
3. All immunocytochemical methods can also be applied to cell culture models.
4. In patients, microglial accumulation can be monitored by PET imaging, using [¹¹C]-PK 11195 as a microglial marker (Banati et al., 2002).
5. Activation of glial cells can be assessed in tissue or cell culture models also by quantification of sets of M1/M2 phenotype markers. This can for instance be done by PCR quantification, immunocytochemistry, immunoblotting.

- Itgam, CD86 expression as markers of M1 microglial phenotype
- Arg1, MRC1, as markers of M2 microglial phenotype

(for descriptions of techniques, see Falsig 2004; Lund 2006; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014)

LIVER:

Kupffer cell activation can be measured by means of expressed cytokines, e.g. tissue levels of TNF-α [Vajdova et al., 2004], IL-6 expression, measured by immunoassays or Elisa (offered by various companies), soluble CD163 [Grønbaek et al., 2012; Møller et al., 2012] or increase in expression of Kupffer cell marker genes such as Lys, Gzmb, and I11b, (Genome U34A Array, Affymetrix); [Takahara et al., 2006]

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Event: 1493: Increased Pro-inflammatory mediators (<https://aopwiki.org/events/1493>)

Short Name: Increased pro-inflammatory mediators

Key Event Component

Process	Object	Action
acute inflammatory response		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory (https://aopwiki.org/aops/17)	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Vertebrates	Vertebrates		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

LIVER:

Human [Santibañez et al., 2011]

Rat [Luckey and Petersen, 2001]

Mouse [Nan et al., 2013]

BRAIN:

Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

Key Event Description

Inflammatory mediators are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites. They can be divided into exogenous and endogenous mediators.

Exogenous mediators of inflammation are bacterial products or toxins like endotoxin or LPS. Endogenous mediators of inflammation are produced from within the (innate and adaptive) immune system itself, as well as other systems. They can be derived from molecules that are normally present in the plasma in an inactive form, such as peptide fragments of some components of complement, coagulation, and kinin systems. Or they can be released at the site of injury by a number of cell types that either contain them as preformed molecules within storage granules, e.g. histamine, or which can rapidly switch on the machinery required to synthesize the mediators.

Table1: a non-exhaustive list of examples for pro-inflammatory mediators

Classes of inflammatory mediators	Examples
Pro-inflammatory cytokines	TNF- α , Interleukins (IL-1, IL-6, IL-8), Interferons (IFN- γ), chemokines (CXCL, CCL, GRO- α , MCP-1), GM-CSF
Prostaglandins	PGE2
Bradykinin	
Vasoactive amines	histamine, serotonin
Reactive oxygen species (ROS)	O ² , H ₂ O ₂
Reactive nitrogen species (RNS)	NO, iNOS

The increased production of pro-inflammatory mediators can have negative consequences on the parenchymal cells leading even to cell death, as described for TNF- α or peroxynitrite on neurons (Chao et al., 1995; Brown and Bal-Price, 2003). In addition, via a feedback loop, they can act on the reactive resident cells thus maintaining or exacerbating their reactive state; and by modifying elements of their signalling pathways, they can favour the M1 phenotypic polarization and the chronicity of the inflammatory process (Taetzsch et al., 2015).

Basically, this event occurs equally in various tissues and does not require tissue-specific descriptions. Nevertheless, there are some specificities such as the release of glutamate by brain reactive glial cells (Brown and Bal-Price, 2003; Vesce et al., 2007). The differences may rather reside in the type of insult favouring the increased expression and/or release of a specific class of inflammatory mediators, as well the time after the insult reflecting different stages of the inflammatory process. For these reasons, the analyses of the changes of a battery of inflammatory mediators rather than of a single one is a more adequate measurement of this KE.

LIVER:

When activated, resident macrophages (Kupffer cells) release inflammatory mediators including cytokines, chemokines, lysosomal, and proteolytic enzymes and are a main source of TGF- β 1 - the most potent pro-fibrogenic cytokine. Following the role of TGF- β is described in more detail.

Transforming growth factor β (TGF- β) is a pleiotropic cytokine with potent regulatory and

inflammatory activity [Sanjabi et al., 2009; Li and Flavell, 2008a;2008b]. The multi-faceted effects of TGF- β on numerous immune functions are cellular and environmental context dependent [Li et al., 2006]. TGF- β binds to TGF- β receptor II (TGF- β RII) triggering the kinase activity of the cytoplasmic domain that in turn activates TGF- β RI. The activated receptor complex leads to nuclear translocation of Smad molecules,

and transcription of target genes [Li et al., 2006a]. The role of TGF- β as an immune modulator of T cell activity is best exemplified by the similarities between TGF- β 1 knockout and T cell specific

TGF- β receptor II knockout mice [Li et al., 2006b; Marie et al., 2006; Shull et al., 1992]. The animals in both of these models develop severe multi-organ autoimmunity and succumb to death within a few weeks after birth [Li et al., 2006b; Marie et al., 2006; Shull et al., 1992]. In addition, in mice where TGF- β signaling is blocked specifically in T cells, the development of natural killer T (NKT) cells, natural regulatory T (nTreg) cells, and CD8+ T cells was shown to be dependent on TGF- β signaling in the thymus [Li et al., 2006b; Marie et al., 2006].

TGF- β plays a major role under inflammatory conditions. TGF- β in the presence of IL-6 drives the differentiation of T helper 17 (Th17) cells, which can promote further inflammation and augment autoimmune conditions [Korn et al., 2009]. TGF- β orchestrates the differentiation of both Treg and Th17 cells in a concentration-dependent manner [Korn et al., 2008]. In addition, TGF- β in combination with IL-4, promotes the differentiation of IL-9- and IL-10-producing T cells, which lack

suppressive function and also promote tissue inflammation [Dardalhon et al., 2008; Veldhoen et al., 2008]. The biological effects of TGF- β under inflammatory conditions on effector and memory CD8+ T cells are much less understood. In a recent study, it was shown that TGF- β has a drastically opposing role on naïve compared to antigen-experienced/memory CD8+ T cells [Filippi et al., 2008]. When cultured *in vitro*, TGF- β suppressed naïve CD8+ T cell activation and IFN- γ production, whereas TGF- β enhanced survival of memory CD8+ T cells and increased the production of IL-17 and IFN- γ [Filippi et al., 2008]. TGF- β also plays an important role in suppressing the cells of the innate immune system.

The transforming growth factor beta (TGF- β) family of cytokines are ubiquitous, multifunctional, and essential to survival. They play important roles in growth and development, inflammation and repair, and host immunity. The mammalian TGF- β isoforms (TGF- β 1, β 2 and β 3) are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction. Autocrine and paracrine effects of TGF- β s can be modified by extracellular matrix, neighbouring cells and other cytokines. The vital role of the TGF- β family is illustrated by the fact that approximately 50% of TGF-1 gene knockout mice die in utero and the remainder succumb to uncontrolled inflammation after birth. The role of TGF- β in homeostatic and pathogenic processes suggests numerous applications in the diagnosis and treatment of various diseases characterised by inflammation and fibrosis. [Clark and Coker, 1998; Santibañez et al., 2011; Pohlers et al., 2009] Abnormal TGF- β regulation and function are implicated in a growing number of fibrotic and inflammatory pathologies, including pulmonary fibrosis, liver cirrhosis, glomerulonephritis and diabetic nephropathy, congestive heart failure, rheumatoid arthritis, Marfan syndrome, hypertrophic scars, systemic sclerosis, myocarditis, and Crohn's disease. [Gordon and Globe, 2008] TGF- β 1 is a polypeptide member of the TGF- β superfamily of cytokines. TGF- β is synthesized as a non-active pro-form, forms a complex with two latent associated proteins latency-associated protein (LAP) and latent TGF- β binding protein (LTBP) and undergoes proteolytic cleavage by the endopeptidase furin to generate the mature TGF- β dimer. Among the TGF- β s, six distinct isoforms have been discovered although only the TGF- β 1, TGF- β 2 and TGF- β 3 isoforms are expressed in mammals, and their human genes are located on chromosomes 19q13, 14q41 and 14q24, respectively. Out of the three TGF- β isoforms (β 1, β 2 and β 3) only TGF- β 1 was linked to fibrogenesis and is the most potent fibrogenic factor for hepatic stellate cells. [Roberts, 1998; Govinden and Bhoola, 2003]. During fibrogenesis, tissue and blood levels of active TGF- β are elevated and overexpression of TGF- β 1 in transgenic mice can induce fibrosis. Additionally, experimental fibrosis can be inhibited by anti-TGF- β treatments with neutralizing antibodies or soluble TGF- β receptors [Qi et al., 1999; Shek and Benyon, 2004; De Gouville et al., 2005; Chen et al., 2009]. TGF- β 1 induces its own mRNA to sustain high levels in local sites of injury. The effects of TGF- β 1 are classically mediated by intracellular signalling via Smad proteins. Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory. [Parsons et al., 2013; Friedman, 2008; Kubiczkova et al., 2012] Smad1/5/8, MAP kinase (mitogen-activated protein) and PI3 kinase are further signalling pathways in different cell types for TGF- β 1 effects.

TGF- β is found in all tissues, but is particularly abundant in bone, lung, kidney and placental tissue. TGF- β is produced by many, but not all parenchymal cell types, and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. Following wounding or inflammation, all these cells are potential sources of TGF- β . In general, the release and activation of TGF- β stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins. [Branton and Kopp, 1999]

TGF- β 1 is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells, and its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of these immune cells. [Letterio and Roberts; 1998]

In the liver TGF- β 1 is released by activated Kupffer cells, liver sinusoidal endothelial cells, and platelets; in the further course of events also activated hepatic stellate cells express TGF- β 1. Hepatocytes do not produce TGF- β 1 but are implicated in intracellular activation of latent TGF- β 1. [Roth et al., 1998; Kisseleva and Brenner, 2007; Kisseleva and Brenner, 2008; Poli, 2000; Liu et al., 2006]

TGF- β 1 is the most established mediator and regulator of epithelial-mesenchymal-transition (EMT) which further contributes to the production of extracellular matrix. It has been shown that TGF- β 1 mediates EMT by inducing snail-1 transcription factor and tyrosine phosphorylation of Smad2/3 with subsequent recruitment of Smad4. [Kolios et al., 2006; Batailler and Brenner, 2005; Guo and Friedman, 2007; Brenner, 2009; Kaimori et al., 2007; Gressner et al., 2002; Kershenobich Stalnikowitz and Weissbrod, 2003; Li et al., 2008; Matsuoka and Tsukamoto, 1990; Kisseleva and Brenner, 2008; Poli, 2000; Parsons et al., 2007; Friedman 2008; Liu et al., 2006]

TGF- β 1 induces apoptosis and angiogenesis in vitro and in vivo through the activation of vascular endothelial growth factor (VEGF) High levels of VEGF and TGF- β 1 are present in many tumors. Crosstalk between the signalling pathways activated by these growth factors controls endothelial cell apoptosis and angiogenesis. [Clark and Coker; 1998]

How it is Measured or Detected

The specific type of measurement(s) might vary with tissue, environment and context and will need to be described for different tissue contexts as used within different AOP descriptions.

In general, quantification of inflammatory markers can be done by:

- PCR (mRNA expression)
- ELISA
- Immunocytochemistry
- Immunoblotting

For descriptions of techniques, see Falsig 2004; Lund 2006 ; Kuegler 2010; Monnet-Tschudi et al., 2011; Sandström et al., 2014; von Tobel et al., 2014

LIVER:

There are several assays for TGB- β 1 measurement available.

e.g. Human TGF- β 1 ELISA Kit. The Human TGF- β 1 ELISA (Enzyme –Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TGF- β 1 in serum, plasma, cell culture supernatants, and urine. This assay employs an antibody specific for human TGF- β 1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TGF- β 1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TGF- β 1 antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour develops in proportion to the amount of TGF- β 1 bound. The StopSolution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm [Mazzieri et al., 2000]

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Event: 1494: Leukocyte recruitment/activation (<https://aopwiki.org/events/1494>)

Short Name: Leukocyte recruitment/activation

Key Event Component

Process	Object	Action
cell activation involved in immune response	leukocyte	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Vertebrates	Vertebrates		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Key Event Description

The inflammatory response is the cornerstone of the body's defense mechanism against bacterial and viral pathogens, as well as physical-, chemical- and environmental-mediated tissue and organ damage. Leukocyte recruitment at the site of pathogen evasion or sterile tissue injury is a critical adaptation for the preservation of tissue integrity. Neutrophils are the cell population that acutely responds to the alterations of inflammatory micro-environment. Neutrophil infiltration takes place within 6-8 hours from the initiation of the inflammatory process and is followed by the recruitment of other cell populations, like monocytes, lymphocytes, and eosinophils, which either promote or drive the resolution of inflammation. Leukocyte infiltration into sites of infection or sterile inflammation is a tightly regulated process that follows a sequence of adhesive events, termed as leukocyte adhesion cascade. One can broadly generalize that most leukocytes follow a similar multi-step cascade in the peripheral (non-lymphoid) vasculature with some exceptions. Accordingly, an updated adhesion cascade in postcapillary venules involves free-flowing leukocytes initial attachment or tethering and slow velocity rolling (step 1), stable adhesion (arrest) on endothelial cells (step 2), leukocyte flattening (step 3), and subsequent crawling on the vascular endothelium, transendothelial cell migration (TEM) between (paracellular route) or through (transcellular) the vascular endothelium (step 4), and uropod elongation to complete transmigration of postcapillary venules (step 5). The initial attachment and rolling steps are initiated by interactions of endothelial E- and P-selectins and their counterreceptors on leukocytes L-selectin (Leick et al., 2014).

Each of these steps is necessary for effective leukocyte recruitment; these steps are not phases of inflammation, but represent the sequence of events from the perspective of each leukocyte. At any given moment they all happen in parallel, involving different leukocytes in the same microvessels.

From the initial selectin-dependent leukocyte tethering to endothelial cells to the final migration of leukocytes into the sub-endothelium, this process depends on the interplay between leukocyte receptors and endothelial cell counter-receptors, as well as on the presence of endogenous inhibitors of leukocyte adhesion enabling the targeted recruitment of leukocytes to inflamed tissues.

To enable the infiltration of leukocytes at the site of inflammation, a series of alterations in endothelial cells and leukocytes takes place:

- regulation of the expression of adhesion molecules in leukocytes
- increased secretion of chemokines by endothelial cells
- increased expression of adhesion molecules in the luminal surface of endothelial cells

(Kourtzelis and Mitroulis, 2015) (Robbins and Cotran: Pathologic Basis of Disease 2010).

After recruitment, activation includes phenotype modification with morphologic alterations, changes in marker proteins (MHC, adhesion molecules, co-stimulatory signal), expression of mediators, enzymes, and pro-inflammatory proteins/lipids. Recruited monocytes recruited mature into macrophages with phagocytic activity and elaboration of a myriad of mediators of inflammation. The macrophage can replicate within tissues or die, including by apoptosis.

How it is Measured or Detected

in vivo imaging:

- Flow cytometry (FC/FACS),
- immunohistochemistry
- two photon-intravital microscopy (TP-IVM) (van Grinsven et al., 2017)
- Spinning Disk Confocal Microscopy-IVM (Jenne et al., 2011)
- Histology, increased cell numbers and altered composition

In vitro

- transwell Migration Assay (Justus et al., 2014)
- T-Lymphocyte & Innate Immune Cell Activation Assays
- Leukocyte Surface Markers (Monoclonal Antibodies to Leukocyte Surface Markers)
- Markers of leukocyte activation – protease release, ROS/RNS, NADPH oxidase (NOX), defense response - expression of anti-oxidants.
- organs-on-a-chip (Bnam et al., 2016; Ribas et al., 2017; Wufuer et al. 2016)

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List of Adverse Outcomes in this AOP

Event: 1194: Increase, DNA damage (<https://aopwiki.org/events/1194>)

Short Name: Increase, DNA Damage

Key Event Component

Process	Object	Action
	deoxyribonucleic acid	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent
Aop:216 - Excessive reactive oxygen species production leading to oocyte apoptosis-associated reproduction decline (https://aopwiki.org/aops/216)	KeyEvent
Aop:266 - D1 protein blockage leading to apoptosis associated growth inhibition (https://aopwiki.org/aops/266)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	MolecularInitiatingEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	AdverseOutcome

Stressors

Name
Ionizing Radiation
Estrogen

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Stressors include:

Ionizing radiation

Estrogen

Ionizing Radiation

When ionizing radiation enters a cell and interacts with cellular components including double stranded DNA, it releases energy that leads to DNA damage. This energy ejects electrons from atoms and molecules, and these electrons can produce more electrons, directly ionize DNA, or radiolyze water to form hydroxyl molecules which damage DNA (Hutchinson 1985; Ward 1988; Ravanat, Breton et al. 2014). DNA damage observed after IR includes oxidized base, sugar (deoxyribose), and phosphate lesions, single and double strand breaks, and cross-linking (Ward 1988; Roots, Holley et al. 1990; Haegele, Wolfe et al. 1998; Pouget, Frelon et al. 2002; Rothkamm and Lobrich 2003). DNA damage from IR can occur in a clustered pattern, even from a single particle or photon (Sutherland, Bennett et al. 2002). The type and amount of DNA damage depends on both the quality and dose of radiation. Higher LET radiation such as alpha particles generates more complex clusters of damage including more frequent double strand breaks (Ottolenghi, Merzagora et al. 1997; Rydberg, Heilbronn et al. 2002; Watanabe, Rahmanian et al. 2015; Nikitaki, Nikolov et al. 2016) and other chromosomal abnormalities (Yang, Georgy et al. 1997; Anderson, Stevens et al. 2002), while lower LET radiation (gamma rays, X-rays) generates more oxidized base damage and single strand breaks (Douki, Ravanat et al. 2006).

Damage is also observed in DNA in cells not directly in the path of ionizing radiation, or at a delay following exposure. Indirect or bystander effects are mediated by multiple factors including RONS (Yang, Asaad et al. 2005), TGF- β (Dickey, Baird et al. 2009), and other cytokines (Havaki, Kotsinas et al. 2015). DNA damage following ionizing radiation in directly and indirectly damaged cells is repaired over the first few hours or days (Nikitaki, Nikolov et al. 2016), but long term DNA damage can reoccur as genomic instability weeks, months, or even years after the initial exposure and persist in subsequent generations of cells in vivo (Pazhanisamy, Li et al. 2011; Datta, Suman et al. 2012; Mukherjee, Coates et al. 2012; Sniijders, Marchetti et al. 2012) and in vitro (Moore, Marsden et al. 2005; Natarajan, Gibbons et al. 2007; Buonanno, de Toledo et al. 2011; Bensimon, Biard et al. 2016).

Double strand breaks occur linearly with dose between 0.001 Gy (the lowest dose at which an effect has been reliably observed) to over 80 Gy in irradiated cells (Rydberg, Heilbronn et al. 2002; Rothkamm and Lobrich 2003; Yang, Asaad et al. 2005; Asaithamby and Chen 2009). Some low dose studies find a steeper slope between 0.001 and 0.01 Gy for X-rays (although not gamma rays), possibly due to underassessment at higher doses or to a bystander effect superimposed on a linear response (Ojima, Ban et al. 2008; Beels, Werbrouck et al. 2010). Clustered DNA damage also occurs linearly from at least 0.05 Gy (the lowest dose tested) (Sutherland, Bennett et al. 2002), and single strand breaks and alkali sensitive lesions are linear with dose in isolated DNA (Roots, Holley et al. 1990). Chromosomal aberrations appear to be linear or supralinear with dose for low LET radiation (Yang, Georgy et al. 1997; Ryu, Kim et al. 2016) and linear with dose for high LET radiation (Yang, Georgy et al. 1997; Jones, Riggs et al. 2007) at doses examined as low as 0.01 Gy (Schiestl, Khogali et al. 1994; Iwasaki, Takashima et al. 2011). DNA damage measured in bystander cells 1 hour to 3 days after exposure is dose-dependent at low doses (0.001-0.005 Gy), but may approach a maximum between 0.005 and 0.1 Gy (Yang, Anzenberg et al. 2007; Ojima, Ban et al. 2008).

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Estrogen

Metabolites created through the oxidative metabolism of estrogens form pro-mutagenic adducts with guanine and adenine. These adducts rapidly undergo depurination leaving abasic sites that can contribute to point mutations or to double strand breaks and further errors if not correctly repaired prior to replication (Cavaliere, Chakravarti et al. 2006; Savage, Matchett et al. 2014; Yager 2015; Yasuda, Sakakibara et al. 2017). The metabolic cycling of the same estrogen metabolites contributes to the formation of ROS, which can oxidatively damage DNA. However, this does not appear to be the major mechanism of DNA damage by estrogen (Cavaliere, Chakravarti et al. 2006). The creation of estrogen metabolites depend on an imbalance in estrogen synthesis and metabolism. DNA damage and mutation is enhanced under conditions that promote estrogen synthesis or inhibit further metabolism including the inactivation of the DNA-damaging metabolites (Cavaliere, Chakravarti et al. 2006; Yager 2015).

Estrogen can also increase double strand breaks through a transcription and replication-dependent mechanism (Stork, Bocek et al. 2016). Estradiol increases double strand breaks and rearrangements at R-loops (RNA-DNA hybrids with an associated single-stranded DNA) formed at ER-mediated transcription sites. This damage is dependent on Transcription-Coupled Nucleotide Excision Repair and occurs after a delay compared with the ER-independent breaks. This mechanism is a major contributor to overall double strand break formation after estrogen treatment (Stork, Bocek et al. 2016).

Estrogen also affects DNA damage less directly through effects on cell cycle checkpoint regulation and DNA repair mechanisms (Caldon 2014; Li, Chen et al. 2014; Schiewer and Knudsen 2016). It enhances some aspects of the cellular response to DNA damage including enhancing Rad51 recruitment of repair machinery but inhibits others aspects of the response including suppressing multiple regulators of cell cycle checkpoints and delaying complete repair of DSBs (Caldon 2014; Li, Chen et al. 2014). Estrogen also promotes relatively error-prone NHEJ repair mechanisms (Caldon 2014). The net effect promotes survival and replication at the expense of genomic integrity. This effect of estrogen on cell cycle and repair also serves to promote the more direct DNA damaging effects of estrogen, since several of the mechanisms by which estrogen damages DNA require replication before repair is complete (Savage, Matchett et al. 2014; Stork, Bocek et al. 2016). Interestingly, the protection against breast cancer afforded by early parity may at least partially be mediated by a change in the response to estrogen signaling to promote p53 activity and genomic integrity at the cost of proliferation (Jery, Dunphy et al. 2010).

The effect of estrogen on cell cycle machinery is closely linked with the canonical proliferative effect of estrogen. Since replication can create DNA damage through collapse of replicative forks encountering unrepaired sites to form DSBs, the proliferative effect itself promotes DNA damage even in the absence of other mechanisms.

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Key Event Description

DNA nucleotide damage, single, and double strand breaks occur in the course of cellular operations such as DNA repair and replication and can be induced directly and in neighboring "bystander" cells by internal or external stressors like reactive oxygen species, chemicals, and radiation. Ionizing radiation and RONS such as hydroxyl radicals or peroxide can create a range of lesions (a change in molecular structure) in the base of the nucleotide, with guanine particularly vulnerable because of its low redox potential (David, O'Shea et al. 2007). The same stressors can also break the sugar (deoxyribose)-phosphate backbone creating a single strand break. Simultaneous proximal breaks in both strands of DNA form double strand breaks, which are considered to be more destructive and mutagenic than lesions or single strand breaks. Double strand breaks can generate chromosomal abnormalities including changes in chromosomal number, breaks and gaps, translocations, inversions, and deletions (Yang, Craise et al. 1992; Haag, Hsu et al. 1996; Ponnaiya, Cornforth et al. 1997; Yang, Georgy et al. 1997; Unger, Wienberg et al. 2010; Behjati, Gudem et al. 2016; Morishita, Muramatsu et al. 2016).

However, DNA lesions and single strand breaks can also be destructive and mutagenic. Lesions can lead to point mutations (David, O'Shea et al. 2007) or single strand breaks (Regulus, Duroux et al. 2007). Lesions and single strand breaks can also promote the formation of double strand breaks: replication fork collapse and double strand breaks sometimes occur during mitosis when the replisome encounters an unrepaired single strand break (Kuzminov 2001), and clustered lesions and closely opposed single strand breaks can also form double strand breaks (Chaudhry and Weinfeld 1997; Vispe and Satoh 2000; Shiraishi, Shikazono et al. 2017). Complex damage consists of any combination of closely opposed DNA lesions, abasic sites, crosslinks, single, or double strand breaks in proximity. While classically induced by ionizing radiation, there is also evidence that it can be induced by oxidative activity (Sharma, Collins et al. 2016) or even by a single oxidizing particle (Ravanat, Breton et al. 2014). Complex damage is more difficult to repair (Kuhne, Rothkamm et al. 2000; Stenerlow, Hoglund et al. 2000; Pinto, Prise et al. 2005; Rydberg, Cooper et al. 2005).

DNA damage and resulting repair activity can trigger a halt in the cell cycle, cell death (apoptosis), and cause permanent changes to DNA including deletions, translocations, and sequence changes. DNA damage is also associated with an increase in genomic instability - the new appearance of DNA damage including double strand breaks, mutations, and chromosomal damage following repair of initial damage in affected cells or in clonal descendants or neighbors of DNA damaged cells. The mechanism behind this long term DNA damage is not clear, but telomere erosion appears to play a major role (Murnane 2012; Sishc, Nelson et al. 2015). Genomic instability is more common and longer lasting following complex damage (Ponnaiya, Cornforth et al. 1997), and is influenced by multiple factors including variants in DNA repair genes (Ponnaiya, Cornforth et al. 1997; Yu, Okayasu et al. 2001; Yin, Menendez et al. 2012), RONS (Dayal, Martin et al. 2008), estrogen (Kutanzi and Kovalchuk 2013), caspases (Liu, He et al. 2015), and telomeres (Sishc, Nelson et al. 2015).

How it is Measured or Detected

DNA damage can be studied in isolated DNA, fixed cells, or living cells. Types of damage that can be detected include single and double strand breaks, nucleotide damage, complex damage, and chromosomal or telomere damage. The OECD test guideline for DNA synthesis Test No. 486 (OECD 1997) detects nucleotide excision repair, so it will reflect the formation of bulky DNA adducts but not the majority of oxidative damage to nucleotides, which is typically repaired via the Base Excision Repair pathway. The OECD test guideline alkaline comet assay Test No. 489 (OECD 2016) detects single and double strand breaks, including those arising from repair as well as some (alkali sensitive) nucleotide lesions including some lesions from oxidative damage. OECD tests for chromosomal damage and micronuclei Test No. 473, 475, 483, and 487 measure longer term effects of DNA damage but these tests require the damaged cell to subsequently undergo replication (OECD 2016; OECD 2016; OECD 2016). They can therefore reflect a wider range of sources of DNA damage including changes in mitosis. Finally, tests for mutations reveal past DNA damage that resulted in a heritable change, and these are described in the key event 'Increase in Mutation'.

Many other (non-test guideline) techniques have been used to examine specific forms of DNA damage (Table 1). Double strand breaks are commonly reported because of the significant risk attributed to breaks and the relative ease of detecting and quantifying them. Historically, single and double strand breaks were measured using gel electrophoresis, but are now commonly visualized microscopically using fluorescent or other labeled probes for double and single strand break repair such as H2AX and XRCC2. Base lesions can also be detected using labeled probes for base excision repair enzymes, or by chemical methods such as mass spectroscopy. Refinements on these methods can be used to characterize complex or clustered damage, in which various forms of damage occur in close proximity on a DNA molecule (Lorat, Timm et al. 2016; Nikitaki, Nikolov et al. 2016).

Certain challenges are common to all methods of detecting DNA damage. In the time required to initiate the detection method, some DNA may already be repaired, leading to undercounting of damage. On the other hand, apoptotic DSBs may be incorrectly included in a measurement of direct (non-apoptotic) induction of DSB damage unless controlled. All methods have difficulty distinguishing individual components of clustered lesions, and microscopic methods may undercount disparate breaks that are processed together in repair centers (Barnard, Bouffler et al. 2013). Methods that use isolated DNA (gel electrophoresis, analytical chemistry) are vulnerable to artifacts and must ensure that the DNA sample is protected from oxidative damage during extraction (Pernot, Hall et al. 2012; Barnard, Bouffler et al. 2013; Ravanat, Breton et al. 2014).

Table 1. Common methods of detecting DNA damage

Target	Name	Method	Strengths/Weaknesses
Nucleotide damage	Single cell gel electrophoresis (comet assay) with restriction enzymes (Collins 2004)	Gel electrophoresis	A variant of the comet assay in which restriction enzymes allow the identification of different types of nucleotide damage. The comet assay is more sensitive than PFGE, detecting damage from 0.1 Gy ionizing radiation (Pernot, Hall et al. 2012). A reproducible high-throughput application of the assay is available (Ge, Prasongtanakij et al. 2014; Sykora, Witt et al. 2018), and the test requires only a small (single cell) sample. Requires destruction of the cell.
Nucleotide damage	Labeled probes including Biotin OxyDNA and anti-8-oxoguanine-DNA glycosylase (OGG1) for oxidative damage and AP endonuclease (APE1) for Base Excision Repair of less bulky lesions such as oxidative damage.	Microscopy, FACS	Most useful with FACS or other measures of average or relative intensity, as locations and numbers of damaged nucleotides can be difficult to distinguish using fluorescence microscopy. (Ogawa, Kobayashi et al. 2003; Nikitaki, Nikolov et al. 2016).
Nucleotide damage	High performance liquid chromatography (HPLC), tandem mass spectrometry (MS/MS)	Analytical chemistry	Capable of quantifying low levels of specific nucleotide lesions (Madugundu, Cadet et al. 2014; Ravanat, Breton et al. 2014). Requires destruction of the cell.
Nucleotide damage	Unscheduled DNA synthesis test OECD Test Guideline 486 (OECD 1997)	Autoradiography	Measures DNA damage that is repaired using Nucleotide Excision Repair - mostly bulky adducts (OECD (Organisation for Economic Co-operation and Development) 2016).
Non-specific DNA strand breaks	Single cell gel electrophoresis (comet assay), alkali conditions OECD Test Guideline 489 (OECD 2016)	Gel electrophoresis	When used in alkali conditions, the comet assay reveals single and double strand breaks and alkali-sensitive nucleotide lesions. See single cell gel electrophoresis (comet assay) with restriction enzymes above for further comments.
Single strand breaks	Labeled probe pXRCC1 (Lorat, Brunner et al. 2015)	Microscopy	Fluorescent probes can label single strand breaks in cells, while immunogold labeling is able to distinguish multiple single strand breaks in clusters (Lorat, Timm et al. 2016; Nikitaki, Nikolov et al. 2016).
Double strand breaks	Single cell gel electrophoresis (comet assay), neutral conditions	Gel electrophoresis	Neutral conditions help minimize the release of single strand breaks coiled DNA and alkali lesions, allowing the measurement of double strand breaks. Since single strand breaks can still appear, assay is not very sensitive or specific to double strand breaks (Pernot, Hall et al. 2012). See single cell gel electrophoresis (comet assay) with restriction enzymes above for further comments.

Double strand breaks	Pulsed field gel electrophoresis (PFGE)	Gel electrophoresis	Permits the quantitative measurement of double strand breaks, and can be combined with immunoblotting to detect DNA-associated proteins (Lobrich, Rydberg et al. 1995; Kawashima, Yamaguchi et al. 2017). Considered less sensitive than comet assay, but detected damage from 0.25 Gy ionizing radiation (Gradzka and Iwanenko 2005). Requires destruction of the cell.
Double strand breaks	Labeled probes including phosphorylated H2AX, 53BP1, Ku70, ATM (Lorat, Brunner et al. 2015)	Microscopy	Fluorescent probes can label individual double breaks in cells allowing for quantification, with immunogold labeling resolving breaks in clusters (Lorat, Timm et al. 2016; Nikitaki, Nikolov et al. 2016). Sensitive: detects damage from 0.001 Gy ionizing radiation (Rothkamm and Lobrich 2003; Ojima, Ban et al. 2008).
Chromosomal damage	Chromosomal aberrations and micronuclei OECD Test Guidelines 473, 475, 483, and 487 (OECD 2016; OECD 2016; OECD 2016; OECD 2016)	Microscopy	Detects major DNA damage resulting from large breaks and rearrangements, or mitotic failures. Damage does not appear until DNA undergoes mitosis, so slower and limited to damage in replicating cells. Insensitive to small deletions and substitutions.

Regulatory Significance of the AO

DNA damage increases the susceptibility to and probability of subsequent mutations, described in the key event 'Increase in Mutation'. Mutations can impair the functional capacity of the cell and are an endpoint of regulator significance in their own right.

Multiple guideline toxicity tests exist for DNA damage. The OECD test guideline for DNA synthesis Test No. 486 (OECD 1997) detects nucleotide excision repair, so it will reflect the formation of bulky DNA adducts but not the majority of oxidative damage to nucleotides, which is typically repaired via the Base Excision Repair pathway. The OECD test guideline alkaline comet assay Test No. 489 (OECD 2016) detects single and double strand breaks, including those arising from repair as well as some (alkali sensitive) nucleotide lesions including some lesions from oxidative damage. OECD tests for chromosomal damage and micronuclei Test No. 473, 475, 483, and 487 measure longer term effects of DNA damage but these tests require the damaged cell to subsequently undergo replication (OECD 2016; OECD 2016; OECD 2016; OECD 2016). They can therefore reflect a wider range of sources of DNA damage including changes in mitosis.

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Event: 185: Increase, Mutations (<https://aopwiki.org/events/185>)

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 (https://aopwiki.org/aops/15)	KeyEvent
Aop:141 - Alkylation of DNA leading to cancer 2 (https://aopwiki.org/aops/141)	KeyEvent
Aop:139 - Alkylation of DNA leading to cancer 1 (https://aopwiki.org/aops/139)	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	AdverseOutcome
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	AdverseOutcome
Aop:296 - Oxidative DNA damage leading to chromosomal aberrations and mutations (https://aopwiki.org/aops/296)	AdverseOutcome
Aop:272 - Direct deposition of ionizing energy onto DNA leading to lung cancer (https://aopwiki.org/aops/272)	KeyEvent

Stressors

Name
Ionizing Radiation

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
medaka	Oryzias latipes	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Homo sapiens	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Mutations can occur in any organism and in any cell type, and are the fundamental material of evolution. The test guidelines described above range from analysis from prokaryotes, to rodents, to human cells in vitro. Mutations have been measured in virtually every human tissue sampled in vivo.

Key Event Description

A mutation is a change in DNA sequence. Mutations can thus alter the coding sequence of genes, potentially leading to malformed or truncated proteins. Mutations can also occur in promoter regions, splice junctions, non-coding RNA DNA segments, and other functional locations in the genome. These mutations can lead to various downstream consequences, including alterations in gene expression. There are several different types of mutations including missense, nonsense, insertion, deletion, duplication, and frameshift mutations, all of which can impact the genome and its expression in unique ways.

Mutations can be propagated to daughter cells upon cellular replication. Mutations in stem cells (versus terminally differentiated non-replicating cells) are the most concerning, as these will persist in the organism. The consequence of the mutation, and thus the fate of the cell, depends on the location (e.g., coding versus non-coding) and the type (e.g., nonsense versus silent) of mutation.

Mutations can occur in somatic cells or germ cells (sperm or egg).

How it is Measured or Detected

Mutations can be measured using a variety of both OECD and non-OECD mutagenicity tests. Some examples are given below.

Somatic cells: The Salmonella mutagenicity test (Ames Test) is generally used as part of a first tier screen to determine if a chemical can cause gene mutations. This well-established test has an OECD test guideline (TG 471). A variety of bacterial strains are used, in the presence and absence of a metabolic activation system (e.g., rat liver microsomal S9 fraction), to determine the mutagenic potency of chemicals by dose-response analysis. A full description is found in Test No. 471: Bacterial Reverse Mutation Test (OECD).

A variety of in vitro mammalian cell gene mutation tests are described in OECD's Test Guidelines 476 and 490. TG 476 is used to identify substances that induce gene mutations at the hprt (hypoxanthine-guanine phosphoribosyl transferase) gene, or the transgenic xprt (xanthine-guanine phosphoribosyl transferase) reporter locus. The most commonly used cells for the HPRT test include the CHO, CHL and V79 lines of Chinese hamster cells, L5178Y mouse lymphoma cells, and TK6 human lymphoblastoid cells. The only cells suitable for the XPRT test are AS52 cells containing the bacterial xprt (or gpt) transgene (from which the hprt gene was deleted).

The new OECD TG 490 describes two distinct in vitro mammalian gene mutation assays using the thymidine kinase (tk) locus and requiring two specific tk heterozygous cells lines: L5178Y tk+/-3.7.2C cells for the mouse lymphoma assay (MLA) and TK6 tk+/- cells for the TK6 assay. The autosomal and heterozygous nature of the thymidine kinase gene in the two cell lines enables the detection of cells deficient in the enzyme thymidine kinase following mutation from tk+/- to tk-/-.

It is important to consider that different mutation spectra are detected by the different mutation endpoints assessed. The non-autosomal location of the hprt gene (X-chromosome) means that the types of mutations detected in this assay are point mutations, including base pair substitutions and frameshift mutations resulting from small insertions and deletions. Whereas, the autosomal location of the transgenic xprt, tk, or gpt locus allows the detection of large deletions not readily detected at the hemizygous hprt locus on X-chromosomes. Genetic events detected using the tk locus include both gene mutations (point mutations, frameshift mutations, small deletions) and large deletions.

The transgenic rodent mutation assay (OECD TG 488) is the only assay capable of measuring gene mutation in virtually all tissues in vivo. Specific details on the rodent transgenic mutation reporter assays are reviewed in Lambert et al. (2005, 2009). The transgenic reporter genes are used for detection of gene mutations and/or chromosomal deletions and rearrangements resulting in DNA size changes (the latter specifically in the lacZ plasmid and Spi-test models) induced in vivo by test substances (OECD, 2009, OECD, 2011; Lambert et al., 2005). Briefly, transgenic rodents (mouse or rat) are exposed to the chemical agent sub-chronically. Following a manifestation period, genomic DNA is extracted from tissues, transgenes are rescued from genomic DNA, and transfected into bacteria where the mutant frequency is measured using specific selection systems.

The Pig-a (phosphatidylinositol glycan, Class A) gene on the X chromosome codes for a catalytic subunit of the N-acetylglucosamine transferase complex that is involved in glycosylphosphatidylinositol (GPI) cell surface anchor synthesis. Cells lacking GPI anchors, or GPI-anchored cell surface proteins are predominantly due to mutations in the Pig-a gene. Thus, flow cytometry of red blood cells expressing or not expressing the Pig-a gene has been developed for mutation analysis in blood cells from humans, rats, mice, and monkeys. The assay is described in detail in Dobrovolsky et al. (2010). Development of an OECD guideline for the Pig-a assay is underway. In addition, experiments determining precisely what proportion of cells expressing the Pig-a mutant phenotype have mutations in the Pig-a gene are in progress (e.g., Nicklas et al., 2015, Drobovolsky et al., 2015). A recent paper indicates that the majority of CD48 deficient cells from 7,12-dimethylbenz[*a*]anthracene-treated rats (78%) are indeed due to mutation in Pig-a (Drobovolsky et al., 2015).

Germ cells: Tandem repeat mutations can be measured in bone marrow, sperm, and other tissues using single-molecule PCR. This approach has been applied most frequently to measure repeat mutations occurring in sperm DNA. Isolation of sperm DNA is as described above for the transgenic rodent mutation assay, and analysis of tandem repeats is done using electrophoresis for size analysis of allele length using single-molecule PCR. For expanded simple tandem repeat this involved agarose gel electrophoresis and Southern blotting, whereas for microsatellites sizing is done by capillary electrophoresis. Detailed methodologies for this approach are found in Yauk et al. (2002) and Beal et al. (2015).

Mutations in rodent sperm can also be measured using the transgenic reporter model (OECD TG 488). A description of the approach is found within this published TG. Further modifications to this protocol have now been made for the analysis of germ cells. Detailed methodology for detecting mutant frequency arising in spermatogonia is described in Douglas et al. (1995), O'Brien et al. (2013); and O'Brien et al. (2014). Briefly, male mice are exposed to the mutagen and killed at varying times post-exposure to evaluate effects on different phases of spermatogenesis. Sperm are collected from the vas deferens or caudal epididymis (the latter preferred). Modified protocols have been developed for extraction of DNA from sperm.

A similar transgenic assay can be used in transgenic medaka (Norris and Winn, 2010).

Please note, gene mutations that occur in somatic cells in vivo (OECD Test No. 488) or in vitro (OECD Test No. 476: In vitro Mammalian Cell Gene Mutation Test), or in bacterial cells (i.e., OECD Test No. 471) can be used as an indicator that mutations in male pre-meiotic germ cells may occur for a particular agent (sensitivity and specificity of other assays for male germ cell effects is given in Waters et al., 1994). However, given the very unique biological features of spermatogenesis relative to other cell types, known exceptions to this rule, and the small database on which this is based, inferring results from somatic cell or bacterial tests to male pre-meiotic germ cells must be done with caution. That mutational assays in somatic cells may predict mutations in germ cells has not been rigorously tested empirically (Singer and Yauk, 2010). The IWGT working group on germ cells specifically addressed this gap in knowledge in their report (Yauk et al., 2015) and recommended that additional research address this issue. Mutations can be directly measured in humans (and other species) through the application of next-generation sequencing. Although single-molecule approaches are growing in prevalence, the most robust approach to measure mutation using next-generation sequencing today requires clonal expansion of the mutation to a sizable proportion (e.g., sequencing tumours; Shen et al., 2015), or analysis of families to identify germline derived mutations (reviewed in Campbell and Eichler, 2013; Adewoye et al., 2015).

Please refer to the table below for additional details and methodologies for measuring mutations.

Assay Name	References	Description	OECD Approved Assay
Assorted Gene Loci Mutation Assays	Tindall et al., 1989; Kruger (file:///ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radiobiology/Vinita/AOP/assay%20summary%20table%20papers/MANY%20OTHER%20gene%20loci%20example%20.pdf) 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 (file:///ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radiobiology/Vinita/AOP/assay%20summary%20table%20papers/TK%20mutation%20assay%20use.pdf) et al., 2017; Liber (file:///ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radiobiology/Vinita/AOP/assay%20summary%20table%20papers/TK%20mutation%20assay%20protocol.pdf) 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 lethalfurorothymidine (TFT); Only TK-/- cells are able to form colonies	Yes (No. 490)
HPRT Mutation Assay	Ayres (file:///ncr-a_hecsbc6s/hecsbc6/share/CCRPB/Radiobiology/Vinita/AOP/assay%20summary%20table%20papers/HPRT%20mutation%20assay%20use.pdf) 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 (file:///ncr- a_hecsbc6s/hecsbc6/share/CCRPB/Radiology/Vinita/AOP/assay%20summary%20table%20papers/MANY%20OTHER%20gene%20loci%20example%202.pdf 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	Kraysberg, 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 polonies	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: 1192: Increased, Ductal Hyperplasia (<https://aopwiki.org/events/1192>)

Short Name: Increased, Ductal Hyperplasia

Key Event Component

Process	Object	Action
hyperplasia		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	KeyEvent
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	AdverseOutcome
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	AdverseOutcome

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
mammary duct

Event: 1193: N/A, Breast Cancer (<https://aopwiki.org/events/1193>)

Short Name: N/A, Breast Cancer

Key Event Component

Process	Object	Action
	Breast Neoplasms	pathological

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:200 - Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	AdverseOutcome
Aop:293 - Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	AdverseOutcome
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Key Event Description

Cancers are thought to arise from a collection of permissive factors which interact within and between different cells of a tissue or tumor to promote tumor growth and invasive characteristics (Sonnenschein and Soto 1999; Hanahan and Weinberg 2011; Floor, Dumont et al. 2012; Goodson, Lowe et al. 2015; Schwarzman, Ackerman et al. 2015; Smith, Guyton et al. 2016; Grashow, De La Rosa et al. 2018). Permissive factors or hallmarks include changes to the cell's dependence on growth signals, proliferation, metabolism, apoptosis, senescence, angiogenesis, and invasion and metastasis. These hallmarks are modified by other factors including growth factors, inflammation, oxidative stress, changes to the microenvironment, DNA damage, and changes in gene expression.

The mammary gland is a hormone responsive organ with multiple phases of development from embryogenesis into adulthood. Consequently, certain hallmarks and contributing factors including proliferative response to growth signals, growth factors, changes to the microenvironment, and changes in gene expression play a larger role in this organ, and the importance of various factors shifts depending on developmental stage (Rudel, Fenton et al. 2011). Established risk factors of breast cancer extend beyond genetic contributors (principally alterations in DNA damage response genes) and DNA damaging environmental agents to include exposure to pharmaceutical hormones, timing of puberty and first birth, and lifetime exposure to estrogen and progesterone ((IOM) Institute of Medicine 2012).

Hormonal and other environmental influences during proliferation and differentiation alter the pace and structure of cellular or mammary gland development to leave tissue in the adult gland more susceptible to cancer. In addition, the elevated hormone concentrations associated with the menstrual cycle and pregnancy provide a regular proliferative stimulus to any pre-cancerous cells present in the breast (Rudel, Fenton et al. 2011). A substantial majority of breast cancers express hormone receptors, and these cancers are particularly responsive to hormones (Badowska-Kozakiewicz, Patera et al. 2015).

Consistent with the importance of growth factors and DNA damage in the development of cancer, driver mutations (mutations that favor the success of the nascent cancer cells and are therefore selected) commonly appear in the growth factor related signaling pathways (BRAF, EGFR, RAS, PI3K, STK11) and in DNA damage response and cell cycle checkpoint signal pathways (ATM, TP53, CHEK2, CDKN2B (P15), CDK4) (Greenman, Stephens et al. 2007; Croce 2008; Kaufmann, Nevis et al. 2008; Stratton, Campbell et al. 2009; Vandin, Uplal et al. 2012). These and other mutations are acquired over the development of a cancer and contribute to the evolution of the cancer (Wang, Waters et al. 2014; Yates, Gerstung et al. 2015; Begg, Ostrovskaya et al. 2016).

In breast cancer, TP53, PI3K and GATA3 are each mutated in more than 10% of cancers, amplification or mutation of the RB1 pathway are common, and HER2 (an EGFR receptor) is amplified in HER2 type cancers (CGAN 2012). EGFR, HER2, BRAF, RAS, and PI3K participate in the EGFR (growth factor) signaling pathway. Activating mutations in PI3K generate growth factor independent proliferation of mammary epithelial cells, possibly via the RB1 pathway (Gustin, Karakas et al. 2009). GATA is a transcription factor that maintains luminal epithelial cell differentiation and suppresses proliferation, and mutation results in the proliferation of undifferentiated cells (Kouros-Mehr, Slorach et al. 2006; Shahi, Wang et al. 2017).

Environmental factors contribute significantly to the total number of breast cancers. Women exposed to the synthetic hormone DES or the pesticide DDT in utero are up to two to four times more likely to be diagnosed with breast cancer in their fifties (Palmer, Wise et al. 2006; Cohn, La Merrill et al. 2015). A study in 2002 found that recipients of hormone replacement therapy (HRT) around menopause are 26% more likely to be diagnosed with breast cancer (Narod 2011). When prescriptions of HRT began to fall in response to the study, so did cancer diagnoses. Over the next few years, approximately 5% fewer cancers were diagnosed in women over 45 (Glass, Lacey et al. 2007) with an estimated 126,000 fewer cases of breast cancer over the next ten years (Roth, Etzioni et al. 2014).

How it is Measured or Detected

In rodent bioassays, tumors can be detected via visual observation or palpation of live animals, necropsy of dead animals, and via microscopic examination of tissue. Malignant tumors including carcinomas in situ are distinguishable from benign tumors on the basis of the thickness or shape of the epithelial cell layer, regularity of the lumen or the presence of cribriform luminae, inflammation or desmoplastic reaction of the stroma, dominance of a less differentiated cell type, and larger nuclei, while diagnosis of invasiveness depends on the identification of metastases or invasion of neoplastic cells into surrounding tissue (Russo and Russo 2000).

In humans, lumps are commonly detected by palpation or mammogram. Further imaging, biopsy, and/or surgical excision of the affected tissue are used to differentiate benign, cancerous, and invasive tumors (McDonald, Clark et al. 2016).

Regulatory Significance of the AO

Because of the long latency of mammary tumors, the two-year rodent carcinogenicity bioassay is the primary assay for this adverse outcome. The assay is included in the OECD Test No. 451 and 453 for carcinogenicity and combined toxicity and carcinogenicity (OECD 2009; OECD 2009), and is also used by the US National Toxicology program (Chhabra, Huff et al. 1990), and the FDA (FDA (Food and Drug Administration) 2007), and referenced by the EPA (EPA (Environmental Protection Agency) 2005) in guidelines for risk assessments. Other assays from short term (2-4 weeks) and subchronic (90 day) to chronic (1 year) toxicity also call for the documentation of mammary tumors (FDA (Food and Drug Administration) 2007; OECD (Organisation for Economic Cooperation and Development) 2018), so these assays could capture the early onset of tumors, and could be modified to report earlier key events like proliferation and inflammation.

Several characteristics of classic cancer bioassays limit the sensitivity of these assays to mammary gland carcinogens. First, no assays require prenatal or early post-natal exposures for carcinogenicity testing. The US NIH's National Toxicology Program assays start exposures at five to six weeks of age and OECD regulatory assay exposures suggest (but do not require) exposures beginning after weaning and before eight weeks of age. Assays initiating exposures at later ages have diminished sensitivity to agents that affect breast development and increase future susceptibility to cancer, such as estrogenic hormones, DDT and dioxins (EPA (Environmental Protection Agency) 2005; Rudel, Fenton et al. 2011). Agents with similar activity to ionizing radiation and DNA damaging chemicals may not be fully captured in some of these assays, since sensitivity appears to peak around or before week seven for these agents (around puberty) (Imaoka, Nishimura et al. 2013). Second, carcinogenicity assay guidelines do not require the best methods for detecting tumors in mammary gland: whole mount preparations of mammary gland coupled with longitudinal sections (dorsoventral sections parallel to the body) of mammary gland for histology (Tucker, Foley et al. 2017). Palpation and transverse sections of mammary gland can easily miss tumors or lesions of interest. Interestingly the NTP reproductive toxicity guidelines do specify these preferable methods for mammary gland analysis.

Two additional factors affect the sensitivity of standard carcinogenicity assays. First, benign tumors are not always considered to be an indicator of carcinogenicity, leading to a possible underestimation of risk. NTP and EPA guidance suggest that benign tumors provide additional weight of evidence if malignant tumors are also present or if studies suggest benign tumors can progress to carcinogenicity. In a short-term study, benign tumors may indicate a need for a longer-term study. However, benign mammary tumors (fibroadenomas) almost always coincide with carcinogenic tumors in mammary gland or other organs, and carcinomas sometimes grow from fibroadenomas (Rudel, Attfield et al. 2007; Russo 2015) suggesting that benign tumors may be an underutilized indicator of carcinogenicity.

Finally, the dose selection guidance in carcinogenicity testing typically calls for a high dose that is sufficiently toxic to suppress body weight (OECD 2009). However, body weight interacts with risk of breast cancer (Haseman, Young et al. 1997; Rudel, Attfield et al. 2007), reducing the sensitivity of the upper end of the dose range and the likelihood of a positive dose-response.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1904: Increase in RONS leads to Increase, DNA Damage (<https://aopwiki.org/relationships/1904>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	High	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified

Key Event Relationship Description

Increased RONS leads to an increase in DNA damage.

Evidence Supporting this KER

Biological plausibility is High. *Reactive oxygen and nitrogen species from oxygen and respiratory activity are generally acknowledged to damage DNA under a range of cellular conditions.*

Empirical support is High. *Multiple studies show an increase in DNA damage with RONS treatment as well as dependent changes in both RONS and DNA damage in response to stressors. DNA damage increases with RONS dose, and temporal concordance between RONS and DNA damage events following ionizing radiation is consistent with a causative relationship, although few studies examine multiple doses and time points. A small number of studies do not find double strand breaks at physiological doses, or report an increase in one key event but not the other.*

Biological Plausibility

High. *Reactive oxygen and nitrogen species from oxygen and respiratory activity are generally acknowledged to damage DNA under typical cellular conditions* (Dickinson and Chang 2011; Aziz, Nowshen et al. 2012; Tubbs and Nussenzweig 2017). Damage commonly occurs via oxidation of a nucleotide by the hydroxyl radical (or by radicals created by nitric oxide), or can occur indirectly in nearby nucleotides following the secondary reaction of a radical created in nucleotides (Cadet, Davies et al. 2017). Oxidative damage predominantly consists of DNA lesions (structural modifications to nucleotides) including single strand breaks, although double strand breaks can occur when transcription or translation machinery encounters damaged strands (Tubbs and Nussenzweig 2017).

Empirical Evidence

High. *Multiple studies show an increase in DNA damage with RONS treatment as well as dependent changes in both RONS and DNA damage in response to stressors. DNA damage increases with RONS dose, and temporal concordance between RONS and DNA damage events following ionizing radiation is consistent with a causative relationship, although few studies examine multiple doses and time points. A small number of studies do not find double strand breaks at physiological doses, or report an increase in one key event but not the other.*

Treatment with H₂O₂ or other RONS induces increase DNA damage and double strand breaks. H₂O₂ treatment reaches the nucleus where it can damage DNA (Ameziane-EI-Hassani, Boufraqueh et al. 2010; Ameziane-EI-Hassani, Talbot et al. 2015). Oxidized nucleotides (including clusters) and single strand breaks are commonly reported following H₂O₂ treatment (Dahm-Daphi, Sass et al. 2000; Nakamura, Purvis et al. 2003; Yang, Durando et al. 2013; Sharma, Collins et al. 2016), and double strand breaks can occur when transcription and translation machinery encounters damaged strands (Berdelle, Nikolova et al. 2011; Yang, Durando et al. 2013; Tubbs and Nussenzweig 2017). However, it is less clear whether H₂O₂ or RONS cause a measurable increase in double strand breaks, particularly at physiologically relevant concentrations (in the range of 12 μM) (Liu and Zweier 2001; Ameziane-EI-Hassani, Talbot et al. 2015). Studies report double strand breaks following treatment with 15 μM- 1mM H₂O₂ (Oya, Yamamoto et al. 1986; Driessens, Versteheyte et al. 2009; Seager, Shah et al. 2012; Werner, Wang et al. 2014; Ameziane-EI-Hassani, Talbot et al. 2015; Sharma, Collins et al. 2016) as well as parallel increases in RONS and double strand breaks (Han, Chen et al. 2010; Berdelle, Nikolova et al. 2011; Stanicka, Russell et al. 2015). DNA damage including double strand breaks and mutations increase with H₂O₂ dose (Sandhu and Birnboim 1997; Dahm-Daphi, Sass et al. 2000; Driessens, Versteheyte et al. 2009; Seager, Shah et al. 2012; Lorat, Brunner et al. 2015; Sharma, Collins et al. 2016).

RONS is dose-dependently and reversibly associated with increased genomic instability (Dayal, Martin et al. 2008; Dayal, Martin et al. 2009; Buonanno, de Toledo et al. 2011; Pazhanisamy, Li et al. 2011; Datta, Suman et al. 2012; Bensimon, Biard et al. 2016) and with DNA damage in bystander cells (Azzam, De Toledo et al. 2002; Yang, Asaad et al. 2005; Yang, Anzenberg et al. 2007; Han, Chen et al. 2010; Buonanno, de Toledo et al. 2011) although other non-RONS factors such as telomere erosion and breakage-fusion-bridge events may be sufficient to maintain genomic instability (Suzuki, Kashino et al. 2009; Murnane 2012). To our knowledge no experiments have tested whether elevating intracellular RONS alone in one group of cells can cause DNA damage in nearby cells.

Antioxidants and other interventions to reduce RONS production also reduce or block the effect of RONS treatment on DNA base damage (Berdelle, Nikolova et al. 2011) and double-strand breaks (Ameziane-EI-Hassani, Boufraqueh et al. 2010; Ameziane-EI-Hassani, Talbot et al. 2015; Stanicka, Russell et al. 2015). Similarly, nitric oxide scavengers can reduce DNA damage in cells treated with nitric oxide producers (Han, Chen et al. 2010) or in bystander cells.

Further support for a causative relationship between RONS and DNA damage comes from many studies showing that antioxidants and other interventions capable of reducing RONS can also reduce DNA damage following IR. Antioxidant reduction of nucleotide damage from IR occurs in isolated DNA (Winyard, Faux et al. 1992; Douki, Ravanat et al. 2006), and in vitro and in vivo antioxidants reduce nucleotide damage, double strand breaks, micronuclei, chromosomal damage, and mutations when added before (Azzam, De Toledo et al. 2002; Choi, Kang et al. 2007; Jones, Riggs et al. 2007; Ameziane-EI-Hassani, Boufraqueh et al. 2010; Ozyurt, Cevik et al. 2014; Ameziane-EI-Hassani, Talbot et al. 2015; Fetisova, Antoschina et al. 2015; Manna, Das et al. 2015), or in the case of delayed (15 min to days) or bystander DNA damage, added after radiation (Yang, Asaad et al. 2005; Han, Chen et al. 2010; Pazhanisamy, Li et al. 2011; Ameziane-EI-Hassani, Talbot et al. 2015). Interestingly, NO specific blockers reduce DNA damage and mutations in bystander cells but not in directly IR cells, suggesting that NO specifically contributes to the bystander effect (Zhou, Ivanov et al. 2008; Han, Chen et al. 2010).

Temporal concordance between RONS and DNA damage events following a stressor (ionizing radiation) is consistent with a causative relationship between RONS and DNA damage. Following ionizing radiation, an increase in RONS typically occurs coincident with DNA damage. Few studies examine multiple doses and time points, and detection methods have differing sensitivities. However, both RONS and double strand breaks appear rapidly after IR (Ameziane-EI-Hassani, Boufraqueh et al. 2010; Denissova, Nasello et al. 2012; Martin, Nakamura et al. 2014), and in several studies RONS and DNA single and double strand breaks, chromosomal damage, and micronuclei appear at the same time points over several days following IR (Choi, Kang et al. 2007; Jones, Riggs et al. 2007; Du, Gao et al. 2009; Saenko, Cieslar-Pobuda et al. 2013; Ameziane-EI-Hassani, Talbot et al. 2015; Manna, Das et al. 2015). RONS also appears coincident with longer term DNA damage including nucleotide damage, double strand breaks, and micronuclei, both in IR exposed (Dayal, Martin et al. 2008; Pazhanisamy, Li et al. 2011; Datta, Suman et al. 2012; Werner, Wang et al. 2014; Ameziane-EI-Hassani, Talbot et al. 2015) and in bystander cells not directly exposed to IR (Buonanno, de Toledo et al. 2011).

Uncertainties and Inconsistencies

While the bulk of the evidence support a mechanism where RONS increases DNA damage, including double strand DNA breaks, not all studies report these effects. Some studies report the induction of single strand breaks by H₂O₂, but only show double strand breaks with H₂O₂ doses at or above 1 mM H₂O₂ (Dahm-Daphi, Sass et al. 2000; Lorat, Brunner et al. 2015) or do not find an effect of H₂O₂ on double strand breaks at any concentration (Gradzka and Iwanenko 2005; Ismail, Nystrom et al. 2005). These conflicting results may be partially explained by experimental variations including temperature (two of the studies showing reduced or no effect were exposed to H₂O₂ at 4°C or colder) or other factors including catalysts required to transform H₂O₂ into DNA damaging OH radicals (Nakamura, Purvis et al. 2003). The reduction of IR-induced DNA damage (including double strand breaks) by antioxidants is strong evidence for an essential role of RONS in DNA damage, but antioxidants don't reduce all DNA damage from IR and anti-oxidants that reduce double strand breaks and chromosomal aberrations after IR don't necessarily reduce baseline DNA damage (Fetisova, Antoschina et al. 2015). This incomplete effect suggests either that antioxidants are unable to fully reduce endogenous RONS, or that additional sources of DNA damage are also at work. Furthermore, RONS can be observed following IR in the absence of DNA nucleotide damage (Yoshida, Goto et al. 2012) and counter to expectations lower (10 μM) doses of H₂O₂ applied six days after IR were associated with a decrease in detectable micronuclei (Werner, Wang et al. 2014), suggesting that additional factors (such as repair and apoptosis or changes in endogenous antioxidants) may influence the effect of RONS on IR-induced DNA damage. Finally, double strand breaks and chromosomal damage can be observed following IR in the absence of measured RONS (Suzuki, Kashino et al. 2009), although since antioxidants are still capable of reducing DNA damage in the absence of measurable RONS, such a discrepancy might be attributable to a lack of sensitivity in RONS detection methods (Yang, Asaad et al. 2005).

Quantitative Understanding of the Linkage

Known Feedforward/Feedback loops influencing this KER

RONS activates or is essential to many inflammatory pathways including TGF- β (Barcellos-Hoff and Dix 1996; Jobling, Mott et al. 2006), TNF (Blaser, Dostert et al. 2016), Toll-like receptor (TLR) (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006; Miller, Goodson et al. 2017; Cavallion 2018), and NF- κ B signaling (Gloire, Legrand-Poels et al. 2006; Morgan and Liu 2011). These interactions principally involve ROS, but RNS can indirectly activate TLRs and possibly NF- κ B. Since inflammatory signaling and activated immune cells can also increase the production of RONS, positive feedback and feedforward loops can occur (Zhao and Robbins 2009; Ratikan, Micewicz et al. 2015; Blaser, Dostert et al. 2016).

Damage inflicted by RONS on cells activate TLRs and other receptors to promote release of cytokines (Ratikan, Micewicz et al. 2015). For example, oxidized lipids or oxidative stress-induced heat shock proteins can activate TLR4 (Miller, Goodson et al. 2017; Cavallion 2018).

ROS is essential to TLR4 activation of downstream signals including NF- κ B. Activation of TLR4 promotes the surface expression and movement of TLR4 into signal-promoting lipid rafts (Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006). This signal promotion requires NADPH-oxidase and ROS (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006). ROS is also required for the TLR4/TRAFF6/ASK-1/p38 dependent activation of inflammatory cytokines (Matsuzawa, Saegusa et al. 2005). ROS therefore amplifies the inflammatory process.

RONS can also fail to activate or actively inhibit inflammatory pathways, and the circumstances determining response to RONS are not well known (Gloire, Legrand-Poels et al. 2006).

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Relationship: 1897: Increase, DNA Damage leads to Increase, Mutations (<https://aopwiki.org/relationships/1897>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	High	Not Specified

Key Event Relationship Description

Mutations occur in one of two major ways: incorporation of an incorrect nucleotide leading to a point mutation, and incorrect rejoining of a double strand break leading to a deletion or other sequence change, homozygosity, or chromosomal damage. Mutations in surviving cells are then propagated to daughter cells.

Evidence Supporting this KER

Biological Plausibility is High. DNA damage in the form of nucleotide damage, single strand and double strand breaks, and complex damage can generate mutations, particularly when a damaged cell undergoes replication.

Empirical Support is High. It is generally accepted that DNA damage leads to mutations. Empirical support comes in part from the observation that agents which increase DNA damage also cause mutations, that DNA damage precedes the appearance of mutations, and that interventions that reduce DNA damage also reduce mutations. None of the identified studies measure both outcomes over the same range of time points. This constitutes a readily addressable data gap.

Biological Plausibility

High. DNA damage in the form of nucleotide damage, single strand and double strand breaks, and complex damage can generate mutations, particularly when a damaged cell undergoes replication.

Nucleotide damage

Damage to single nucleotides can generate mutations. Oxidative damage and ionizing radiation can induce a range of base lesions, but guanine is particularly vulnerable because of its low redox potential (David, O'Shea et al. 2007). Repair is generally accurate, but generates single strand breaks. Repair processes can also insert an incorrect nucleotide where a lesion has been excised. If not corrected by mismatch repair processes before a replication cycle, an incorrect base is matched with its pair and is made permanent (Tubbs and Nussenzweig 2017). In a cell undergoing replication, the replication fork typically stalls at a lesion until repairs are complete, but translesion synthesis allows the replication fork to proceed at the cost of increased errors, or mutations (Abbotts and Wilson 2017). Different lesions vary in their frequency and ability to escape repair or be replicated and incorrectly paired by DNA polymerase, making some lesions more mutagenic than others. For example, guanine lesion 8-oxoguanine is very common, so although it is efficiently repaired it contributes to guanine mutations. Other guanine lesions including Fapy and hydantoin are less common but very mutagenic, so likely also contribute to guanine mutations (Neeley and Essigmann 2006; David, O'Shea et al. 2007). Thymine glycol is another common oxidative lesion formed from thymine that can also generate mutations.

Single strand breaks

Single strand breaks are generally repaired efficiently through a variant of the base excision repair pathway. However, replication fork collapse can occur when the replisome encounters an unrepaired single strand break, resulting in a double strand break (Kuzminov 2001).

Double strand breaks

Double strand breaks can generate mutations ranging from point mutations to inversions, deletions, duplications, and chromosomal gaps, breaks, and micronuclei. Double strand breaks can be repaired via two to three major pathways depending on damage type and cell stage among other conditions, and the mutation type and frequency depends on the repair mechanism employed.

Double strand breaks generated from the stalling or collapse of replication forks around lesions or single strand break are processed using homologous recombination (HR) (Rothkamm and Lobrich 2003; Ceccaldi, Rondinelli et al. 2016). Because these breaks happen during replication, an identical sister chromatid may be present to use as a template and repair can restore the original sequence. However, HR can also occur using a non-sister chromatid or in the case of repeated regions can use another stretch of DNA as a template, resulting in loss of homozygosity, inversions, deletions, and duplications (Saleh-Gohari, Bryant et al. 2005; Shrivastav, De Haro et al. 2008). HR may also increase point mutations (Shrivastav, De Haro et al. 2008).

Double strand breaks occurring in all parts of the cell cycle may be processed by non-homologous end joining (NHEJ) (Rothkamm and Lobrich 2003), which can alter the nucleotide sequence of the two broken ends to achieve a fusible template leading to point mutations, deletions, and insertions (Ceccaldi, Rondinelli et al. 2016). NHEJ can fuse incorrect ends within or between chromosomes, resulting in major changes including translocations, deletions, inversions, and duplications. Compared with HR, NHEJ is considered to be more likely to generate mutations, particular the resection dependent classical or alternative end joining pathways (Ceccaldi, Rondinelli et al. 2016).

Complex damage

Complex damage delays repair and increases double strand breaks, increasing the likelihood of mutation. Clustered lesions or single strand breaks are processed more slowly than non-clustered lesions, increasing the number of lesions that will undergo replication and potentially generate mutations in daughter cells (Dianov, Timchenko et al. 1991; Eccles, O'Neill et al. 2011). Clustered damage made of closely opposed lesions and/or single strand breaks can also create double strand breaks (Chaudhry and Weinfeld 1997; Vispe and Satoh 2000; Yang, Galick et al. 2004; Schipler and Iliakis 2013; Sharma, Collins et al. 2016; Shiraishi, Shikazono et al. 2017). Complex damage involving double strand breaks is also repaired more slowly (Stenerlow, Hoglund et al. 2000; Schipler and Iliakis 2013; Lorat, Timm et al. 2016), and undergoes a form of NHEJ with excision that leads to increased translocations and deletions (Eccles, O'Neill et al. 2011; Sharma, Collins et al. 2016; Watts 2016).

Genomic Instability/Long term effects

Genomic instability is the prolonged appearance of DNA damage, chromosomal damage, and mutations. It is sometimes seen following agents that induce DNA damage including ionizing radiation, RONS, and NMU (Goepfert, Moreno-Smith et al. 2007; Kadhim, Salomaa et al. 2013; Stanicka, Russell et al. 2015). DNA damage occurring during genomic instability is associated with the appearance of mutations including deletions, inversions, and duplications (Murnane 2012; Kadhim, Salomaa et al. 2013; Sishc, Nelson et al. 2015).

Empirical Evidence

High. It is generally accepted that DNA damage leads to mutations. Empirical support comes in part from the observation that agents which increase DNA damage also cause mutations, that DNA damage precedes the appearance of mutations, and that interventions that reduce DNA damage also reduce mutations. None of the identified studies measure both outcomes over the same range of time points. This constitutes a readily addressable data gap.

Ionizing radiation (IR) and reactive oxygen and nitrogen species (RONS) are both capable of causing DNA damage including lesions, single and double strand breaks, and these agents also cause mutations (Schiestl, Khogali et al. 1994; Kuhne, Rothkamm et al. 2000; Rydberg, Cooper et al. 2005; Dayal, Martin et al. 2008; Seager, Shah et al. 2012), and chromosomal aberrations (Choi, Kang et al. 2007; Jones, Riggs et al. 2007; Du, Gao et al. 2009; Buonanno, de Toledo et al. 2011; Patil, Rao et al. 2014; Fetisova, Antoschina et al. 2015; Padula, Ponzinibbio et al. 2016). DNA damage observed after IR and RONS precedes the appearance of mutations (Denissova, Nasello et al. 2012; Sharma, Collins et al. 2016) and precedes or is concordant with the appearance of chromosomal aberrations (Yang, Asaad et al. 2005; Du, Gao et al. 2009; Suzuki, Kashino et al. 2009; Patil, Rao et al. 2014; Padula, Ponzinibbio et al. 2016). Further evidence that mutations arise from DNA damage comes from agents that affect DNA repair: decreasing repair after IR maintains damage while decreasing translocations (mutations introduced by DNA repair) (Biehs, Steinlage et al. 2017).

Uncertainties and Inconsistencies

Despite the generally accepted relationship between DNA damage and mutations, few studies uncovered in the literature for RONS or ionizing radiation measure both DNA damage and mutations in the same study (Denissova, Nasello et al. 2012; Sharma, Collins et al. 2016; Biehs, Steinlage et al. 2017) and none measure both key events at the same time points.

Quantitative Understanding of the Linkage

Response-response relationship

Mutations generally increase linearly with dose of DNA damaging agents (Sandhu and Birnboim 1997; Sharma, Collins et al. 2016), but multiple factors including DNA repair, bystander effects, and genomic instability can affect the shape of the dose-response. IR promotion of DNA repair mechanisms decrease major mutations (lethal recessive changes) at lower IR doses/dose rates in flies (0.2 Gy at 0.05 Gy/min gamma) (Koana and Tsujimura 2010). In contrast, non-targeted effects of IR contribute to supralinear responses at lower doses (Sandhu and Birnboim 1997; Hall and Hei 2003; Yang, Anzenberg et al. 2007). At higher doses (10-80 Gy) rearrangements from misrejoining (joining together of non-sequential DNA) increase linearly with dose for high LET IR, but supralinearly for low LET IR, attributed to the increase in the concentration and complexity of double strand breaks with LET (Rydberg, Cooper et al. 2005).

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Relationship: 1896: Increase, Mutations leads to Increase, Cell Proliferation (Epithelial Cells) (<https://aopwiki.org/relationships/1896>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified

Key Event Relationship Description

Mutations altering gene expression or protein activity can enable cells to escape growth inhibition by increasing resistance to apoptosis, or other inhibitory signals, or by escape of cell cycle checkpoints. Alternatively, mutations can stimulate growth by activating proliferative pathways such as EGFR.

Evidence Supporting this KER

Biological plausibility is High. Multiple mechanisms limit the proliferation of cells in healthy biological systems. Mutations in many of the genes controlling these mechanisms promote proliferation.

Empirical support is Moderate. Mutations that promote proliferation are frequently found in cancers, and both mutation and proliferation occur in response to tumorigenic stressors like ionizing radiation. Mutations appear over the same time frame or prior to the appearance of proliferation. Multiple uncertainties and conflicting evidence weaken this key event relationship. The two key events differ in their dose response- mutation but not proliferation increases with ionizing radiation dose. Furthermore, a single mutation is not necessarily sufficient to increase proliferation- proliferation typically requires multiple mutations or a change in the surrounding environment. In mammary tissue, stromal state – which is modified by hormones - strongly influences the proliferative nature of epithelial cells, and mutated epithelial cells alone appear to be insufficient for tumor growth.

Biological Plausibility

High. Multiple mechanisms limit the proliferation of cells in healthy biological systems. Mutations in many of the genes controlling these mechanisms promote proliferation. Biological mechanisms such as contact inhibition, apoptosis, cell cycle checkpoints, and growth factor availability act to restrain proliferation (Sonnenschein and Soto 1999). Under conditions of proliferation such as ductal branching during development of the mammary gland, selected mechanisms are engaged to permit controlled or directed proliferation. In the case of ductal branching, stromal cells respond to estrogen and growth hormone by releasing IGF1, which activates IGF-1R in epithelial cells to promote survival and proliferation (Hinck and Silberstein 2005; Sternlicht, Sunnarborg et al. 2005; Sternlicht 2006). At puberty, epithelial cells respond to estrogen by signaling to the stroma via EGFR to which the stroma replies with proliferative signals via FGFR (Sternlicht, Sunnarborg et al. 2005; Sternlicht 2006). Multiple additional mechanisms of control include proliferation inhibition by TGF- β , which can both directly inhibit proliferation (Francis, Bergsied et al. 2009) and act through stromal cells to stabilize an inhibitory extracellular matrix (Hinck and Silberstein 2005). When mechanisms controlling proliferation are altered, proliferation can occur outside of the normal biological context (Radice, Ferreira-Cornwell et al. 1997; Davies, Platt-Higgins et al. 1999; Ewan, Shyamala et al. 2002; Lanigan, O'Connor et al. 2007; Croce 2008; de Ostrovich, Lambertz et al. 2008).

Empirical Evidence

Moderate. Mutations that promote proliferation are frequently found in cancers, and both mutation and proliferation occur in response to tumorigenic stressors like ionizing radiation. Mutations appear over the same time frame or prior to the appearance of proliferation. Multiple uncertainties and conflicting evidence weaken this key event relationship. The two key events differ in their dose response- mutation but not proliferation increases with ionizing radiation dose. Furthermore, a single mutation is not necessarily sufficient to increase proliferation- proliferation typically requires multiple mutations or a change in the surrounding environment. In mammary tissue, stromal state – which is modified by hormones - strongly influences the proliferative nature of epithelial cells, and mutated epithelial cells alone appear to be insufficient for tumor growth.

Gene sequencing performed on a wide range of cancers has revealed common mutations (Alexandrov, Nik-Zainal et al. 2013). Some mutations are particularly common in certain cancers, while others are more widely observed. Driver mutations commonly appear in EGFR and other tyrosine kinase signaling pathways (BRAF, EGFR, RAS, PI3K, STK11) and in DNA damage response and cell cycle checkpoint signal pathways (ATM, TP53, CHEK2, CDKN2B (P15), CDK4) (Greenman, Stephens et al. 2007; Croce 2008; Kaufmann, Nevis et al. 2008; Stratton, Campbell et al. 2009; Vandin, Uptal et al. 2012). The same processes are commonly affected in breast cancer. PIK3CA (part of PI3K) is commonly mutated, and HER2 (an EGFR receptor) is amplified in HER2 type cancers. Amplification or mutations of the RB1 checkpoint control pathway and dysfunction in BRCA and homologous recombination related DNA repair processes are also common (CGAN 2012; Nik-Zainal, Davies et al. 2016; Davies, Glodzik et al. 2017).

While the presence of these mutations in cancers does not prove causality, in vitro studies confirm that these mutations interfere with signals controlling proliferation, and that the mutations increase proliferation, hyperplasias, and tumors (Podsypanina, Politi et al. 2008). Cell cycle regulatory proteins ATM and P53 respond to DNA damage or other growth inhibiting or senescence signals like TGF- β by limiting entry into the cell cycle via RB1 - a process controlled by CHEK2, CDKN2B, CDK4, and other factors - and are also involved in activating apoptosis. Mutations in these checkpoint related genes enable cells to escape mechanisms limiting proliferation in vitro (Tao, Roberts et al. 2011; Higashiguchi, Nagatomo et al. 2016) and mammary hyperplasia in vivo (Francis, Bergsied et al. 2009), and contribute to tumors when combined with other mutations in the same pathway (Francis, Chakrabarti et al. 2011).

EGFR, HER2, BRAF, RAS, and PI3K participate in the EGFR (growth factor) signaling pathway. Activating mutations in PI3K generates growth factor independent proliferation of mammary epithelial cells, possibly via the RB1 pathway (Gustin, Karakas et al. 2009). GATA is a transcription factor that maintains luminal epithelial cell differentiation and suppresses proliferation, and mutation results in the proliferation of undifferentiated cells (Kouros-Mehr, Slorach et al. 2006; Shahi, Wang et al. 2017).

While no research directly links specific mutations from IR and chemical DNA-damaging agents with subsequent proliferation and hyperplasia in the same experiment, multiple studies support an increase in proliferation and hyperplasia following exposure to these mutagenic stressors. Proliferative nodules and hyperplasia appear in mammary terminal end bud, alveolae, and ducts of rats and mice after exposure to chemical carcinogens (Beuing, Faulkin et al. 1967; Russo, Saby et al. 1977; Purnell 1980) and ionizing radiation (Faulkin, Shellabarger et al. 1967; Ullrich and Preston 1991; Imaoka, Nishimura et al. 2006). Based on the time frame of mutation and proliferation measured in different tissue following exposure to IR, mutations precede or occur over the same general timeframe as proliferation and hyperplasia, consistent with a causative mechanism (Ullrich and Preston 1991; Schiestl, Khogali et al. 1994; Sandhu and Bimboim 1997; Wu, Randers-Pehrson et al. 1999; Zhou, Ivanov et al. 2005; Imaoka, Nishimura et al. 2006; Liang, Deng et al. 2007; Ameziane-El-Hassani, Boufraqech et al. 2010; Datta, Hydeuk et al. 2012; Snijders, Marchetti et al. 2012; Suman, Johnson et al. 2012; Tang, Fernandez-Garcia et al. 2014; Fibach and Rachmilewitz 2015; Sherborne, Davidson et al. 2015; Zhou, Ma et al. 2015). However, proliferation after IR has also been reported within the first day, before the likely appearance of mutations (Han, Chen et al. 2010; Cho, Kang et al. 2016).

Uncertainties and Inconsistencies

Mutations are clearly not the only events driving proliferation in mammary gland, particularly in female mammary glands after exposure to a stressor like ionizing radiation where proliferation varies with age and microenvironment (Tang, Fernandez-Garcia et al. 2014). In mammary tissue, stromal state strongly influences the proliferative and metastatic nature of epithelial cells, and mutated epithelial cells alone appear to be insufficient for tumor growth. Stroma exposed to carcinogens can make transplanted unexposed epithelial cells tumorigenic in rats (Maffini, Soto et al. 2004) and transplanted p53 mutant epithelial cells tumorigenic in BALB/c mice (Barcellos-Hoff and Ravani 2000), while neither epithelia exposed to carcinogens nor p53 mutant cells are tumorigenic when transplanted into unexposed animals (Barcellos-Hoff and Ravani 2000; Maffini, Soto et al. 2004). Similarly, post-lactational stroma can make tumor cells more invasive and metastatic than nulliparous stroma (McDaniel, Rumer et al. 2006), and younger and nulliparous stroma makes tumor cells proliferate more than older and multiparous stroma (Maffini, Calabro et al. 2005). Even proliferating tissue and tumors can regress (Haslam and Bern 1977; Purnell 1980), suggesting that proliferation is insufficient for carcinogenesis in some cases.

While mutations increase linearly in response to ionizing radiation or carcinogens, proliferation (or proliferation of stem cell populations) apparently does not (Beuing, Bern et al. 1967; Mukhopadhyay, Costes et al. 2010; Nguyen, Oketch-Rabah et al. 2011; Tang, Fernandez-Garcia et al. 2014). Because we expect only a subset of mutations to affect cell-cycle or proliferation-related genes and because most cells require multiple mutations for proliferation to commence, only a very small number of cells would be expected to proliferate in response to mutation. It is therefore possible that the proliferation typically observed is actually due to a separate mechanism such as the self-renewal of stem-like or senescent-resistant cells and that a delayed mutation-based proliferation is not being measured.

Quantitative Understanding of the Linkage

Known Feedforward/Feedback loops influencing this KER

Proliferation increases the likelihood that existing DNA damage will result in mutation and creates new mutations through errors in replication.

It is generally accepted that proliferation increases the risk of mutation and cancer (Preston-Martin, Pike et al. 1990). DNA damage that has not been completely or correctly repaired when a cell undergoes mitosis can be fixed in the genome permanently as a mutation, to be propagated to future daughter cells. Incomplete DNA repair can also cause additional DNA damage when encountered by replicative forks. Therefore, in the presence of any DNA damage (and there is a background rate of damage in addition to any other genotoxic stimuli) mutations will increase with cell division (Kiralay, Gong et al. 2015). Mutation-prone double strand breaks can also arise from replicative stress in hyperplastic cells including hyperplasia arising from excess growth factor stimulation (Gorgoulis, Vassiliou et al. 2005). This relationship between proliferation and mutation is thought to drive a significant portion of the risk of cancer from estrogen exposure since breast cells proliferate in response to estrogen or estrogen plus progesterone and risk increases with cumulative estrogen exposure (Preston-Martin, Pike et al. 1990).

Not all proliferating tissue shows replicative stress and DSBs - tissue with a naturally high proliferative index like colon cells don't show any sign of damage (Halazonetis, Gorgoulis et al. 2008). Additional factors are therefore required beyond replication for damage and mutation from replicative stress, but replication is essential for the expression of these factors.

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Relationship: 1907: Increase, Cell Proliferation (Epithelial Cells) leads to Increase, Mutations (<https://aopwiki.org/relationships/1907>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Not Specified	Not Specified

Key Event Relationship Description

Proliferation increases the likelihood that existing DNA damage will result in mutation and creates new mutations through errors in replication.

Evidence Supporting this KER

Biological Plausibility

It is generally accepted that proliferation increases the risk of mutation and cancer (Preston-Martin, Pike et al. 1990). DNA damage that has not been completely or correctly repaired when a cell undergoes mitosis can be fixed in the genome permanently as a mutation, to be propagated to future daughter cells. Incomplete DNA repair can also cause additional DNA damage when encountered by replicative forks. Therefore, in the presence of any DNA damage (and there is a background rate of damage in addition to any other genotoxic stimuli) mutations will increase with cell division (Kivaly, Gong et al. 2015). Mutation-prone double strand breaks can also arise from replicative stress in hyperplastic cells including hyperplasia arising from excess growth factor stimulation (Gorgoulis, Vassiliou et al. 2005). This relationship between proliferation and mutation is thought to drive a significant portion of the risk of cancer from estrogen exposure since breast cells proliferate in response to estrogen or estrogen plus progesterone and risk increases with cumulative estrogen exposure (Preston-Martin, Pike et al. 1990).

Not all proliferating tissue shows replicative stress and DSBs - tissue with a naturally high proliferative index like colon cells don't show any sign of damage (Halazonetis, Gorgoulis et al. 2008). Additional factors are therefore required beyond replication for damage and mutation from replicative stress, but replication is essential for the expression of these factors.

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Kivaly, O., G. Gong, et al. (2015). "Inflammation-induced cell proliferation potentiates DNA damage-induced mutations in vivo." *PLoS Genet* 11(2): e1004901.

Preston-Martin, S., M. C. Pike, et al. (1990). "Increased cell division as a cause of human cancer." *Cancer Res* 50(23): 7415-7421.

Relationship: 1247: Increase, Cell Proliferation (Epithelial Cells) leads to Increased, Ductal Hyperplasia (<https://aopwiki.org/relationships/1247>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	adjacent	High	High
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Not Specified	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Not Specified	Not Specified

Relationship: 1252: Increased, Ductal Hyperplasia leads to N/A, Breast Cancer (<https://aopwiki.org/relationships/1252>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Estrogen receptor activation leading to breast cancer (https://aopwiki.org/aops/200)	adjacent	High	High
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	High	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified

Key Event Relationship Description

Proliferative lesions are believed to evolve over time and with successive cell divisions to take on the hallmarks of carcinogenesis, either directly or via other cell types recruited to the site such as fibroblasts and macrophages.

Evidence Supporting this KER

Biological Plausibility is High. It is generally accepted that proliferation contributes to cancer. Proliferation increases mutations, which can further promote proliferation and/or changes to the local microenvironment.

Empirical support is High. Carcinogenic agents increase proliferation and hyperplasia as well as tumors. Proliferation and hyperplasia appears prior to or at the same time as tumors, grow into carcinomas, and are more effective at forming mammary tumors than non-proliferating tissue. Disruption of proliferation is associated with decreased tumor growth, and tumor resistant rats do not show proliferation. However, the discrepancy between the non-linear proliferative and linear mammary tumor response to carcinogen dose coupled with evidence of independent occurrences of proliferation and tumorigenesis suggests that while proliferation and hyperplasia likely promote carcinogenesis, additional factors also contribute.

Biological Plausibility

Biological Plausibility is High. It is generally accepted that proliferation contributes to cancer. Proliferation increases mutations, which can further promote proliferation and/or changes to the local microenvironment. For example, cells that become insensitive to certain TGF-β signaling pathways would be resistant to contact or TGF-β inhibition (Polyak, Kato et al. 1994) or apoptosis (Chapman, Lourenco et al. 1999), and cells that release or promote the stromal release of MMPs remodel the stroma and promote tumorigenesis and invasiveness (Sternlicht, Lochter et al. 1999; Ha, Moon et al. 2001).

Empirical Evidence

High. Carcinogenic agents increase proliferation and hyperplasia as well as tumors. Proliferation and hyperplasia appears prior to or at the same time as tumors, grow into carcinomas, and are more effective at forming mammary tumors than non-proliferating tissue. Disruption of proliferation is associated with decreased tumor growth, and tumor resistant rats do not show proliferation. However, the discrepancy between the non-linear proliferative and linear mammary tumor response to carcinogen dose coupled with evidence of independent occurrences of proliferation and tumorigenesis suggests that while proliferation and hyperplasia likely promote carcinogenesis, additional factors also contribute.

Factors that increase proliferation or hyperplasia also increase tumors. Proliferative epithelial cells, nodules and hyperplasia appear in mammary gland of rats and mice after exposure to chemical carcinogens (Beuving, Bern et al. 1967; Beuving, Faulkin et al. 1967; Russo, Saby et al. 1977; Purnell 1980) and ionizing radiation (Faulkin, Shellabarger et al. 1967; Ullrich and Preston 1991; Imaoka, Nishimura et al. 2006; Nguyen, Oketch-Rabah et al. 2011; Snijders, Marchetti et al. 2012; Suman, Johnson et al. 2012; Tang, Fernandez-Garcia et al. 2014). A subpopulation of post-senescent epithelial cells also proliferate following IR in vitro (Mukhopadhyay, Costes et al. 2010).

Proliferation and hyperplasia precede or are detected at the same time as tumors (Beuving, Bern et al. 1967; Beuving, Faulkin et al. 1967; Faulkin, Shellabarger et al. 1967; Haslam and Bern 1977; Russo, Saby et al. 1977; Purnell 1980; Imaoka, Nishimura et al. 2005; Imaoka, Nishimura et al. 2006) and form tumors more effectively than non-proliferating tissue (Deome, Faulkin et al. 1959; Beuving 1968; Rivera, Hill et al. 1981). Adenocarcinomas appear to form from terminal end bud hyperplasia in rats (Haslam and Bern 1977; Russo, Saby et al. 1977; Purnell 1980), similar to the origin of many breast cancers for humans and for some mice after IR (Medina and Thompson 2000).

Interrupting or preventing proliferation or hyperplasia reduces the incidence (or growth) of tumors. Disruption of proliferation or hyperplasia formation disrupts tumor growth (Luo, Fan et al. 2009; Connelly, Barham et al. 2011; Tang, Fernandez-Garcia et al. 2014). Similarly, ACI rats exhibit no proliferation or hyperplasia following IR and are resistant to tumors following IR (Shellabarger, Stone et al. 1976; Kutanzi, Koturbash et al. 2010).

Uncertainties and Inconsistencies

In the relatively small number of studies that examine the dose-dependence of proliferation and hyperplasia in models of carcinogenesis, proliferation does not appear to increase linearly with dose (Han, Chen et al. 2010; Mukhopadhyay, Costes et al. 2010; Nguyen, Oketch-Rabah et al. 2011; Tang, Fernandez-Garcia et al. 2014) while tumor formation and carcinogenesis does increase linearly with dose.

Some studies report carcinogenesis in the absence of hyperplasia (Middleton 1965; Sinha and Dao 1974) and others do not find increased tumorigenesis from transplanted hyperplasia (Haslam and Bem 1977; Sinha and Dao 1977). In Copenhagen rats resistant to tumors from MNU treatment, hyperplasia appear after MNU treatment but do not progress into carcinomas in situ, instead disappearing over time (Korkola and Archer 1999). Similarly, Fisher rats are less sensitive to tumor induction by DMBA, and hyperplasia from these rats do not go on to form tumors when transplanted (Beuving, Bem et al. 1967).

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Relationship: 1906: Increase in RONS leads to Tissue resident cell activation (<https://aopwiki.org/relationships/1906>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified

Key Event Relationship Description

Increased RONS leads to an increase in inflammation.

Evidence Supporting this KER

Biological Plausibility is Moderate. RONS can activate some inflammatory and anti-inflammatory pathways (TLR, TGF- β , NF-kB), and RONS are an essential part of multiple inflammatory and anti-inflammatory pathways (TLR4, TNF- α , TGF- β , NF-kB).

Empirical Evidence is Moderate. Both RONS and inflammation increase in response to agents that increase RONS or inflammation, and antioxidants reduce inflammation. Multiple studies show dose-dependent changes in both RONS and inflammation in response to stressors including ionizing radiation and antioxidants. RONS have been measured at the same or earlier time points as inflammatory markers, but additional studies are needed to characterize the inflammatory response at the earliest time points to support causation. Uncertainties come from the positive feedback from inflammation to RONS potentially interfering with attempts to establish causality, and from the large number of inflammation-related endpoints with differing responses to stressors and experimental variation.

Biological Plausibility

Biological Plausibility is Moderate. RONS can activate some inflammatory and anti-inflammatory pathways (TLR, TGF- β , NF-kB), and RONS are an essential part of multiple inflammatory and anti-inflammatory pathways (TLR4, TNF- α , TGF- β , NF-kB).

RONS activates or is essential to many inflammatory pathways including TGF- β (Barcellos-Hoff and Dix 1996; Jobling, Mott et al. 2006), TNF (Blaser, Dostert et al. 2016), Toll-like receptor (TLR) (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006; Miller, Goodson et al. 2017; Cavaillon 2018), and NF-kB signaling (Gloire, Legrand-Poels et al. 2006; Morgan and Liu 2011). These interactions principally involve ROS, but RNS can indirectly activate TLRs and possibly NF-kB. Since inflammatory signaling and activated immune cells can also increase the production of RONS, positive feedback and feedforward loops can occur (Zhao and Robbins 2009; Ratikan, Micewicz et al. 2015; Blaser, Dostert et al. 2016).

Damage inflicted by RONS on cells activate TLRs and other receptors to promote release of cytokines (Ratikan, Micewicz et al. 2015). For example, oxidized lipids or oxidative stress-induced heat shock proteins can activate TLR4 (Miller, Goodson et al. 2017; Cavaillon 2018).

ROS is essential to TLR4 activation of downstream signals including NF-kB. Activation of TLR4 promotes the surface expression and movement of TLR4 into signal-promoting lipid rafts (Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006). This signal promotion requires NADPH-oxidase and ROS (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006). ROS is also required for the TLR4/TRAF6/ASK-1/p38 dependent activation of inflammatory cytokines (Matsuzawa, Saegusa et al. 2005). ROS therefore amplifies the inflammatory process.

RONS can also fail to activate or actively inhibit inflammatory pathways, and the circumstances determining response to RONS are not well known (Gloire, Legrand-Poels et al. 2006).

Empirical Evidence

Empirical Evidence is Moderate. Both RONS and inflammation increase in response to agents that increase RONS or inflammation, and antioxidants reduce inflammation. Multiple studies show dose-dependent changes in both RONS and inflammation in response to stressors including ionizing radiation and antioxidants. RONS have been measured at the same or earlier time points as inflammatory markers, but additional studies are needed to characterize the inflammatory response at the earliest time points to support causation. Uncertainties come from the positive feedback from inflammation to RONS potentially interfering with attempts to establish causality, and from the large number of inflammation-related endpoints with differing responses to stressors and experimental variation.

Oxidative activity is required for or promotes the response to multiple inflammatory stressors, including ionizing radiation, UV radiation (particularly UVB), the endotoxin LPS and other pathogen associated immune activators, and hemorrhagic shock (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006; Zhao and Robbins 2009; Ha, Chung et al. 2010; Hiramoto, Kobayashi et al. 2012; Straub, New et al. 2015).

Both intracellular concentrations of RONS and a wide range of inflammatory markers increase in response to RONS stressors. This paired increase was observed *in vivo* in rodents in tissue from multiple internal organs following exposure to whole body or abdominal ionizing radiation (Berruyer, Martin et al. 2004; Ha, Chung et al. 2010; Sinha, Das et al. 2011; Sinha, Das et al. 2012; Das, Manna et al. 2014; Ozyurt, Cevik et al. 2014; Khan, Manna et al. 2015; Zetner, Andersen et al. 2016; Haddadi, Rezaeayan et al. 2017; Ezz, Ibrahim et al. 2018) or following UV skin irradiation (Sharma, Meeran et al. 2007; Hiramoto, Kobayashi et al. 2012; Martinez, Pinho-Ribeiro et al. 2016). *In vitro*, the relationship has been reported in response to IR and UV in keratinocytes (Park, Ju et al. 2006; Kang, Kim et al. 2007; Martin, Sur et al. 2008; Lee, Jeon et al. 2010; Ren, Shi et al. 2016; Hung, Tang et al. 2017; Zhang, Zhu et al. 2017), immune cells (Matsuzawa, Saegusa et al. 2005; Nakahira, Kim et al. 2006; Manna, Das et al. 2015; Soltani, Ghaemi et al. 2016), as well as corneal and conjunctival epithelia, HEK cells, and vocal cord and foreskin fibroblasts (Narayanan, LaRue et al. 1999; Park, Jung et al. 2004; Saltman, Kraus et al. 2010; Black, Gordon et al. 2011; Han, Min et al. 2015). Direct application of micromolar concentrations of H₂O₂ *in vitro* also increases inflammatory markers in immune cells (Matsuzawa, Saegusa et al. 2005; Nakao, Kurokawa et al. 2008) and keratinocytes (Zhang, Zhu et al. 2017).

Interventions to reduce oxidative activity also reduce inflammation, further implicating RONS in the inflammatory process. Reduction of inflammation by these interventions has been documented in animals in response to IR (Berruyer, Martin et al. 2004; Sinha, Das et al. 2011; Sinha, Das et al. 2012; Das, Manna et al. 2014; Ozyurt, Cevik et al. 2014; Khan, Manna et al. 2015; Zetner, Andersen et al. 2016; Haddadi, Rezaeayan et al. 2017; Ezz, Ibrahim et al. 2018), UV (Sharma, Meeran et al. 2007; Lee, Jeon et al. 2010; Hiramoto, Kobayashi et al. 2012; Han, Min et al. 2015; Martinez, Pinho-Ribeiro et al. 2016; Ren, Shi et al. 2016; Hung, Tang et al. 2017) and hemorrhagic shock (Powers, Szaszzi et al. 2006). *In vitro*, multiple studies in immune cells (Matsuzawa, Saegusa et al. 2005; Nakahira, Kim et al. 2006; Manna, Das et al. 2015; Soltani, Ghaemi et al. 2016) and keratinocytes (Park, Ju et al. 2006; Kang, Kim et al. 2007; Martin, Sur et al. 2008; Lee, Jeon et al. 2010; Ren, Shi et al. 2016; Hung, Tang et al. 2017) as well as HEK293, fibroblasts, and epithelial cells (Lee, Dimtchev et al. 1998; Narayanan, LaRue et al. 1999; Park, Jung et al. 2004; Han, Min et al. 2015) provide further evidence for reduction in various inflammatory markers with interventions to reduce RONS. Interventions include antioxidants such as propyl gallate, *n*-acetylcysteine, or naringin, as well as reduction in the function of NADPH oxidases (NOX/DUOX) via DPI or knockdown of gene expression. In studies using multiple doses of antioxidant, inflammation was reduced dose-dependently with the antioxidant dose (Nakahira, Kim et al. 2006; Manna, Das et al. 2015; Ren, Shi et al. 2016). Interventions reducing nitric oxide were not common, but in one study inhibiting iNOS did not reduce activation of NF- κ B by IR (Lee, Dimtchev et al. 1998). The treatment to reduce RONS is administered before, or occasionally immediately after the inflammatory stressor, but experiments often continue treatment or don't explicitly report changing media *in vitro*, so the exact time point at which RONS are required is difficult to pinpoint.

IR and RONS decrease endogenous antioxidant activity (glutathione, superoxide dismutase, and catalase), and antioxidants rescue this suppression in antioxidant activity (Sharma, Meeran et al. 2007; Das, Manna et al. 2014). Mice with more endogenous glutathione have a lower inflammatory response to IR (Berruyer, Martin et al. 2004), suggesting that IR increases inflammation in part by decreasing antioxidants.

In response to inflammatory stressors, RONS has been measured at the same (Nakao, Kurokawa et al. 2008; Ha, Chung et al. 2010; Saltman, Kraus et al. 2010; Azimzadeh, Scherthan et al. 2011; Ameziane-El-Hassani, Talbot et al. 2015; Azimzadeh, Sievert et al. 2015; Zhang, Zhu et al. 2017) or earlier time points as inflammatory markers (Nakahira, Kim et al. 2006; Black, Gordon et al. 2011). This suggests that RONS precedes the generation of inflammatory markers, consistent with a role for RONS in promoting inflammation. However, inflammatory markers are not typically measured at the earliest time points, and a more comprehensive survey of the appearance of these events at early time points would help to clarify the timeline and confirm the temporal evidence for causation.

A relatively small number of studies in a variety of cell types have examined both RONS and inflammatory markers across multiple doses. Three of these report dose-dependent increases in both RONS and inflammatory markers; one in which the key events are evaluated immediately after H₂O₂ application (Nakao, Kurokawa et al. 2008), and two others evaluating them 24 hours or 8-16 weeks after IR (Ha, Chung et al. 2010; Azimzadeh, Sievert et al. 2015). A fourth study reports a dose-dependent reduction in inflammation in response to treatment with antioxidants (Nakahira, Kim et al. 2006). In three other studies, some or all markers of inflammation increase at lower doses but decrease at higher doses (Saltman, Kraus et al. 2010; Black, Gordon et al. 2011; Zhang, Zhu et al. 2017). In two of these studies, RONS is also not consistently increasing with dose (Saltman, Kraus et al. 2010; Zhang, Zhu et al. 2017), however, this finding is consistent with findings from other studies about lack of dose-dependence of ROS measured at intermediate time points after IR. Similarly, 30 minutes after low dose, IR IL8 increases with dose while ROS does not (Narayanan, LaRue et al. 1999). The mixed inflammatory response at higher doses suggests that additional factors such as negative and positive feedback and crosstalk between pathways are also involved in the relationship between RONS and IR.

Uncertainties and Inconsistencies

Although ROS can activate NF- κ B (Gloire, Legrand-Poels et al. 2006), not all studies consistently show NF- κ B activation after RONS stressor IR. It is possible that the link between ROS and NF- κ B depends on the local environmental context, with different studies not adequately controlling all influential variables. One study offers a possible explanation based on temporal response: in macrophages, NF- κ B was activated by shorter exposures to H₂O₂ (30 min), but the response disappeared with longer exposures (Nakao, Kurokawa et al. 2008).

While many models *in vivo* and *in vitro* showed a decreased inflammatory response to RONS stressors IR in combination with antioxidants, in endothelial cells in culture the increase in IL6 and IL8 after IR was not reduced by antioxidants, although a synergistic increase in those cytokines occurring with combined TNF- α and IR treatment was reduced by antioxidants (Meeren, Bertho et al. 1997). This is a reminder that multiple mechanisms can increase inflammation, that inflammatory factors participate in positive feedback loops, and that responses to stimuli vary between cells.

Quantitative Understanding of the Linkage

Known Feedforward/Feedback loops influencing this KER

Since inflammatory signaling and activated immune cells can also increase the production of RONS, positive feedback and feedforward loops can occur (Zhao and Robbins 2009; Ratikan, Micewicz et al. 2015; Blaser, Dostert et al. 2016).

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Relationship: 1898: Increase, DNA Damage leads to Tissue resident cell activation (<https://aopwiki.org/relationships/1898>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified

Evidence Supporting this KER

Biological Plausibility

High. Biological plausibility is high since DNA damage generates inflammatory signals (DAMPs).

Relationship: 1763: Tissue resident cell activation leads to Increased pro-inflammatory mediators (<https://aopwiki.org/relationships/1763>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Protein Alkylation leading to Liver Fibrosis (https://aopwiki.org/aops/38)	adjacent	High	
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

LIVER:

Human [Battaler and Brenner, 2005; Kolios et al., 2006] (https://aopwiki.org/relationships/147#cite_note-Battaler_2005-4)

Rat [De Bleser et al., 1997] (https://aopwiki.org/relationships/147#cite_note-De_Blaser_1997-11)

Key Event Relationship Description

LIVER:

Following activation the liver resident macrophages, Kupffer cells (KCs), become a major source for inflammatory mediators including cytokines, chemokines, lysosomal, and proteolytic enzymes and for reactive oxygen species (ROS) and also the main source for TGF- β 1, the most potent profibrogenic cytokine. [Luckey and Petersen 2001; Winwood and Arthur 1993]

Expressed TNF- α (Tumor Necrosis Factor -alpha), TRAIL (TNF-related apoptosis-inducing ligand), and FasL (Fas Ligand) are pro-inflammatory active and also capable of inducing death receptor-mediated apoptosis in hepatocytes.

Activated KCs are an important source of ROS like superoxide (generated by NADPH oxidase (NOX)). KCs express TNF- α , IL-1 (Interleukin-1) and MCP-1 (monocyte-chemoattractant protein-1), all being mitogens and chemoattractants for HSCs and induce the expression of platelet-derived growth factor (PDGF) receptors on hepatic stellate cells (HSCs) which further enhances HSCs proliferation. [Kamimura and Tsukamoto, 1995; Li et al., 2008; Kolios et al., 2006; Battaler and Brenner, 2005; Lee and Friedman, 2011; Brenner, 2009; Fujiwara and Kobayashi, 2005; Kirkham, 2007; Reuter et al., 2010]

Evidence Supporting this KER

Biological Plausibility

LIVER:

The functional relationship between these KEs is consistent with biological knowledge. [Kamimura and Tsukamoto, 1995; Li et al., 2008; Kolios et al., 2006; Battaler and Brenner, 2005; Lee and Friedman, 2011; Guo and Friedman, 2007; Brenner, 2009; Fujiwara and Kobayashi, 2005; Kirkham, 2007; Reuter et al., 2010]

Empirical Evidence

LIVER:

Cytokine release is one of the features that define KC activation and there is sound empirical evidence for this KER. Experimental studies have shown enhanced cytokine gene expression by KCs in evolution of experimental liver injury. Northern blot analysis of freshly isolated KCs showed enhanced mRNA expression of three acute phase cytokines by the hepatic resident macrophages, TNF- α , IL-6 and TGF- β . [Kamimura and Tsukamoto, 1995; De Bleser et al., 1997; Chu et al., 2013]

Experiments by Matsuoka and Tsukamoto already 1990 showed that KCs isolated from rat liver with alcoholic fibrosis express and release TGF- β 1 and that this cytokine is largely responsible for the KC-conditioned medium-induced stimulation of collagen formation by HSCs. [Matsuoka and Tsukamoto, 1990]

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LIVER:

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Relationship: 1777: Increased pro-inflammatory mediators leads to Leukocyte recruitment/activation (<https://aopwiki.org/relationships/1777>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Endocytic lysosomal uptake leading to liver fibrosis (https://aopwiki.org/aops/144)	adjacent	High	
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human (Bleul et al., 1996; Miyamoto et al., 2000; Yamada et al., 2001; Sun et al., 2015)

Sheep (Nikiforou et al., 2016)

Mouse (Narumi et al., 1992; Fahy et al., 2001; Lee et al., 2009)

Key Event Relationship Description

Circulating blood leukocytes are required to migrate to sites of injury and infection with the aim to eliminate the primary inflammatory trigger and contribute to tissue repair. In this process are involved selectins (expressed both on leukocytes and endothelium) and integrins (expressed on leukocytes) (von Andrian et al., 1991), with the essential role of the vascular endothelium.

Fast activation of the endothelium with inflammatory stimuli such as histamine and PAF (type I) or slow activation with tumor necrosis factor (TNF) or cytokine interleukin-1 β (IL-1 β) (type II), makes the surface of endothelium adhesive (Bevilacqua and Gimbrone, 1987; Pober and Sessa, 2007). This transformation is mediated by a transcriptionally regulated program involving the nuclear factor NF- κ B dependent pathway triggered by pro-inflammatory cytokines or bacterial endotoxins (reviewed by Collins et al., 1995).

Integrins mediate attachment between cells or to basement membrane. The β 2 integrin family is exclusively expressed on leukocytes and is essential for leukocyte arrest on the endothelium and for migration across the endothelium (Ley et al., 2007). In unstimulated leukocytes integrins are usually in a conformation with low binding affinity, until they receive signals from other receptors, such as chemokine receptors (G-protein-coupled receptors), when they change their conformation and display high affinity for ligands (Luo et al., 2007). Chemokines activate β 1 or β 2 integrins on monocytes, neutrophils, and lymphocytes and as such serve as chemoattractant for these cells during inflammation (Huber et al., 1991; Tanaka et al., 1993; Gunn et al., 1998).

The chemokines are a family of structurally related cytokines that can act as pro-inflammatory agents (Baggiolini et al., 1994; Vaddi et al., 1997). They have the ability to attract leukocyte subsets to specific sites. They recruit neutrophils, monocytes, natural killer cells (NK) and natural killer T (NKT) cells, all of which express inflammatory chemokine receptors and immature dendritic cells (DCs) that provide the link between innate and adaptive immunity (Oo et al., 2010). After antigen-specific activation of lymphocytes by activated DCs, inflammatory chemokines then attract antigen-specific effector T cells to the inflammatory site (Heydtmann and Adams, 2002).

During diapedesis, leukocytes migrate across the endothelium and basement membrane to enter tissue (Ley et al., 2007; Yadav et al., 2003). Once in tissue, the leukocyte follows chemokine gradients to sites of inflammation, using chemokine-mediated changes in the actin cytoskeleton to propel migration. For example, it was demonstrated that chemokines CXCL9, CXCL10 and CXCL11 are important not only in adhesion, but also in transmigration of effectors T lymphocytes through hepatic endothelium (Curbishley et al., 2005; Eksteen et al., 2004). Intracellular actin reorganization is a prerequisite for cell movement, and it has been shown that chemokines such as SDF-1 induce and increase intracellular filamentous actin in lymphocytes (Bleul et al., 1996).

There is essential role of interleukins, but also other factors such as tumor necrosis factor (TNF), interferon (IFN) in leukocyte recruitment and production of chemokines.

Normally, IL-1 β binds to IL-1R1 receptor on the surface of target cells. Following ligand binding the adaptor molecule, myeloid differentiation factor-88 (MyD88), interacts with IL-1R1 via its toll interleukin receptor (TIR) domain (O'Neill, 2008). Signal transduction leads to activation of both mitogen-activated protein kinases (MAPKs) and the transcription factor NF- κ B, and resulting in pro-inflammatory cytokine expression. For example, chemokine RANTES production requires the transcription factor NF- κ B and the activation of mitogen-activated protein kinases (MAPKs) (Genin et al., 2000; Miyamoto et al., 2000; Kujime et al., 2000; Maruoka et al., 2000; Yang et al., 2000).

TNF- α cleavage produces an intracellular domain that translocates to the nucleus and induces pro-inflammatory cytokine signalling, particularly the expression of IL-12 (Friedman et al., 2006). IL-18 induces natural killer and natural killer T cells to produce IFN- γ (Okamura et al., 1998), but it requires IL-12 to induce IFN- γ production by Th1 cells (Nakanishi et al., 2001). There is an essential role of IFN- γ in promoting the chronic recruitment of Ly6Chi monocytes. IFN- γ production is elicited via a toll like receptor-7 (TLR-7) and MyD88-dependent pathway (Lee et al., 2008).

While CXC-chemokines, e.g. IL-8, act mostly on neutrophils (Springer, 1995), members of the CC-chemokines, e.g. RANTES and macrophage inflammatory protein have been shown to exert function on monocytes, eosinophils and lymphocytes (Baggiolini et al., 1994; Carr et al., 1994). This depends on the receptors that are expressed on leukocytes. Th1 express preferentially CCR5 and CXCR3, while Th2 cells have CCR3, CCR4 and CCR8 on their surface (Syrbe et al., 1999). Monocytes and macrophages express CCR5 and other receptors for RANTES (Weber et al., 2000).

RANTES chemokine is produced by many cells in the extravascular compartment, including fibroblasts, epithelial cells, and tissue-infiltrating lymphocytes and monocytes (MacEwan, 2002; Hehlhans and Männel, 2002; Black et al., 1997). It acts as a potent chemoattractant for monocytes, memory T cells, eosinophils, and basophils (Schall et al., 1988, 1990; Baggiolini and Dahinden, 1994). Elevated levels of RANTES transcripts are detected within hours of exposure to pro-inflammatory stimuli, including IL-1 β , TNF- α , IFN- γ , viruses and LPS (Barnes et al., 1996).

Evidence Supporting this KER

Biological Plausibility

There is much evidence that application of chemokines attract leukocytes to specific site in different species (Beck et al., 1997; Lee et al., 2000; Fahy et al., 2001; Nikiforou et al., 2016).

Empirical Evidence

It was shown that a number of chemokines destabilize the rolling of lymphocytes on L-selectin ligands, suggesting that chemokines are capable of regulating the rolling process (Grabovsky et al., 2002).

Jorgensen and colleagues showed that exposure of mice to FltCnd strain S. Typhimurium triggered a significant neutrophil influx in the spleen of wild-type mice, but not Il1b^{-/-}Il18^{-/-} mice (Jorgensen et al., 2016).

The expression of chemokines CCL2, CCL7, and CCL12 was reduced dramatically in MyD88^{-/-} mice (Lee et al., 2009).

Miyamoto and colleagues showed that exposure of cells to IL-1 β , TNF- α , and IFN- γ resulted in the induction of RANTES mRNA and protein (Ortiz et al., 1996; Miyamoto et al., 2000). The levels of RANTES production by the fibroblasts in the presence of IL-1 β or TNF- α were significantly elevated compared with those in the absence of these factors (Yamada et al., 2001).

In the mouse, IFN- γ administration induces high levels of IP-10 expression in liver and kidneys, with lower levels in spleen (Narumi et al., 1992).

CAPE inhibitor of NF- κ B blocked partially IL-1 β induced expression of chemokines MIP-1a and MIP-1b (Guo et al., 2003).

CCR2 and CCR5 receptors on the CD8 T cells are enriched in the inflamed human liver, and CCR1 is important in the regulation of hepatic inflammation in murine models (Shields et al., 1999; Boisvert et al., 2003).

Intradermal injection of RANTES induces a potent T-lymphocyte and eosinophils recruitment (Fahy et al., 2001; Beck et al., 1997). Intradermal administration of MIP-1a resulted in accumulation of monocytes, lymphocytes, eosinophils and recruitment of neutrophils (Lee et al., 2000).

Direct IL-1 α exposure to the gut resulted in increased numbers of CD3⁺ cells in the fetal sheep ileum when compared with control animals on the first day after the exposure. The number of white blood cells, monocytes, and neutrophils was increased in cord blood after 6 days of IL-1 α exposure to the lung and chorioamniot/ skin. The number of lymphocytes on the day 6 was increased for the lung. Compared with controls, gut mRNA levels of TNF- α and IL-1 was significantly increased at 6 days after IL-1 α exposure to the GI tract (Nikiforou et al., 2016).

IL-1 β induced up-regulation of CXCR4 in certain cancer cells, but in order to do so necessary is that these cells have IL-1R1. Presence of IL-1R antagonist significantly inhibited the up-regulation of CXCR4 induced by IL-1 β at both mRNA and protein level (Sun et al., 2015).

SDF-1 is an efficacious chemoattractant and showed a similar dose response for murine lymphocytes and human monocytes, but was not active on human or murine neutrophils. SDF-1 is a highly effective transendothelial chemoattractant (Bleul et al., 1996).

Uncertainties and Inconsistencies

Lloyd and colleagues found that several chemokines can stimulate the adherence of peripheral blood lymphocytes to ICAM-1 coated slides (Lloyd et al., 1996). However, by using a parallel plate flow chamber, other study failed to observe such an effect (Carr et al., 1996).

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Relationship: 1905: Leukocyte recruitment/activation leads to Increase in RONS (<https://aopwiki.org/relationships/1905>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	High	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified

Key Event Relationship Description

Leukocyte recruitment and activation increases reactive oxygen and nitrogen species (RONS).

Evidence Supporting this KER

Biological Plausibility is High. Inflammation is commonly understood to generate RONS via inflammatory signaling and activated immune cells

Empirical Support is High. Signals arising from inflammation can be both pro- and anti-inflammatory, and both can have effects on RONS and downstream key events. Multiple inflammation-related factors increase RONS or oxidative damage, and the stressor ionizing radiation (IR) increases both inflammation-related signaling and RONS or oxidative damage over the same time points. Interventions to reduce inflammation also reduce RONS. The dose-dependence response to stressors is generally consistent between the two key events, although this is based on a small number of studies with some conflicting evidence.

Biological Plausibility

Biological Plausibility is High. Inflammation is commonly understood to generate RONS via inflammatory signaling and activated immune cells (Zhao and Robbins 2009; Ratikan, Micewicz et al. 2015; Blaser, Dostert et al. 2016). Inflammation-related signals contributing to RONS include the cytokines TNF- α , IL1, and INF and the JNK/MAPK pathway (Bubici, Papa et al. 2006; Yang, Elner et al. 2007; Blaser, Dostert et al. 2016), as well as neutrophil and macrophage immune cells (Jackson, Gajewski et al. 1989; Stevens, Bucurenci et al. 1992; Fan, Li et al. 2007; Lorimore, Chrystal et al. 2008; Rastogi, Boylan et al. 2013; Weigert, von Knethen et al. 2018).

Empirical Evidence

Empirical Support is High. Signals arising from inflammation can be both pro- and anti-inflammatory, and both can have effects on RONS and downstream key events. Multiple inflammation-related factors increase RONS or oxidative damage, and the stressor ionizing radiation (IR) increases both inflammation-related signaling and RONS or oxidative damage over the same time points. Interventions to reduce inflammation also reduce RONS. The dose-dependence response to stressors is generally consistent between the two key events, although this is based on a small number of studies with some conflicting evidence.

Multiple inflammation-related factors increase RONS or oxidative damage including neutrophils (Jackson, Gajewski et al. 1989; Stevens, Bucurenci et al. 1992), macrophages (Rastogi, Boylan et al. 2013), TNF- α (Fehsel, Kolb-Bachofen et al. 1991; Yan, Wang et al. 2006; Natarajan, Gibbons et al. 2007; Zhang, Zhu et al. 2017), and TGF- β (Shao, Folkard et al. 2008; Dickey, Baird et al. 2009; Dickey, Baird et al. 2012). Inflammation-related factors TGF- β , TNF- α , COX2, and NO are also implicated in the generation of RONS in bystander cells after IR (Shao, Folkard et al. 2008; Zhou, Ivanov et al. 2008; Wang, Wu et al. 2015).

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Quantitative Understanding of the Linkage

Known Feedforward/Feedback loops influencing this KER

RONS activates or is essential to many inflammatory pathways including TGF- β (Barcellos-Hoff and Dix 1996; Jobling, Mott et al. 2006), TNF (Blaser, Dostert et al. 2016), Toll-like receptor (TLR) (Park, Jung et al. 2004; Nakahira, Kim et al. 2006; Powers, Szaszi et al. 2006; Miller, Goodson et al. 2017; Cavallion 2018), and NF- κ B signaling (Gloire, Legrand-Poels et al. 2006; Morgan and Liu 2011). These interactions principally involve ROS, but RNS can indirectly activate TLRs and possibly NF- κ B.

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Relationship: 1903: Increased pro-inflammatory mediators leads to Increase in RONS (<https://aopwiki.org/relationships/1903>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	High	Not Specified
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	High	Not Specified

Key Event Relationship Description

Pro-inflammatory mediators increase reactive oxygen and nitrogen species (RONS).

Evidence Supporting this KER

Biological Plausibility is High. Inflammation is commonly understood to generate RONS via inflammatory signaling and activated immune cells.

Empirical Support is High. Signals arising from inflammation can be both pro- and anti-inflammatory, and both can have effects on RONS and downstream key events. Multiple inflammation-related factors increase RONS or oxidative damage, and ionizing radiation increases both inflammation-related signaling and RONS or oxidative damage over the same time points. Interventions to reduce inflammation also reduce RONS. The dose-dependence response to stressors is generally consistent between the two key events, although this is based on a small number of studies with some conflicting evidence.

Biological Plausibility

Biological Plausibility is High. Inflammation is commonly understood to generate RONS via inflammatory signaling and activated immune cells (Zhao and Robbins 2009; Ratikan, Micewicz et al. 2015; Blaser, Dostert et al. 2016). Inflammation-related signals contributing to RONS include the cytokines TNF- α , IL1, and INF and the JNK/MAPK pathway (Bubici, Papa et al. 2006; Yang, Elner et al. 2007; Blaser, Dostert et al. 2016), as well as neutrophil and macrophage immune cells (Jackson, Gajewski et al. 1989; Stevens, Bucurenci et al. 1992; Fan, Li et al. 2007; Lorimore, Chrystal et al. 2008; Rastogi, Boylan et al. 2013; Weigert, von Knethen et al. 2018).

Empirical Evidence

High. Signals arising from inflammation can be both pro- and anti-inflammatory, and both can have effects on RONS and downstream key events. Multiple inflammation-related factors increase RONS or oxidative damage, and ionizing radiation increases both inflammation-related signaling and RONS or oxidative damage over the same time points. Interventions to reduce inflammation also reduce RONS. The dose-dependence response to stressors is generally consistent between the two key events, although this is based on a small number of studies with some conflicting evidence.

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Relationship: 1908: Increased pro-inflammatory mediators leads to Increase, Cell Proliferation (Epithelial Cells) (<https://aopwiki.org/relationships/1908>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified

Key Event Relationship Description

Pro-inflammatory factors increase proliferation.

Evidence Supporting this KER

Biological Plausibility is High. Inflammation is generally understood to lead to proliferation during recovery from inflammation.

Biological Plausibility

Biological Plausibility is High. Inflammation is generally understood to lead to proliferation during recovery from inflammation. Major inflammation-related transcription factors NF-kB, AP1, and STAT3 mediate proliferation and survival (Grivennikov, Greten et al. 2010). Inflammation enhances compensatory proliferation during wound healing including following inflammation-associated cell killing (Landen, Li et al. 2016), and contributes to proliferation via growth enhancing signals including changes to the microenvironment and promotion of stem-like characteristics (Grivennikov, Greten et al. 2010; Hanahan and Weinberg 2011; Kiraly, Gong et al. 2015). These effects can vary between tissues and in different contexts in ways that are not well understood (Grivennikov, Greten et al. 2010). In mammary gland and in mammary epithelial cells, inflammation-related transcription factor NF-kB and multiple cytokines promote proliferation and tumorigenesis (Connelly, Barham et al. 2011; Esquivel-Velazquez, Ostoa-Saloma et al. 2015).

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Relationship: 1902: Increased pro-inflammatory mediators leads to N/A, Breast Cancer (<https://aopwiki.org/relationships/1902>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer (https://aopwiki.org/aops/294)	adjacent	Moderate	Not Specified
Increased DNA damage leading to increased risk of breast cancer (https://aopwiki.org/aops/293)	adjacent	Moderate	Not Specified

Key Event Relationship Description

Pro-inflammatory mediators increase the risk of breast cancer.

Evidence Supporting this KER

Biological Plausibility is Moderate. Tissue environment is known to be a major factor in carcinogenesis, and inflammatory processes are implicated in the development and invasiveness of breast and other cancers.

Empirical Evidence is Moderate. Interventions to increase inflammatory factors increase the carcinogenic potential of targeted and non-targeted cells. Inflammation is documented at earlier time points than tumorigenesis or invasion- within minutes or hours compared to days to months for carcinogenesis, consistent with an inflammatory mechanism of tumorigenesis and invasion. Inhibition of cytokines, inflammatory signaling pathways, and downstream effectors of inflammation activity prevent transformation, tumorigenesis, and invasion (including EMT and senescence) following IR or stimulation of inflammatory pathways. However, the key event and the adverse outcome differ in their dose-response to ionizing radiation: inflammation does not increase linearly with dose, while breast cancer and invasion does. Uncertainty arises from differences between the CBA/Ca mouse susceptible to leukemia from IR and the BALB/c mouse susceptible to mammary tumors from IR- the former has a pro-inflammatory response while the latter is apparently a mix of anti- and pro-inflammatory. This is a reminder that both pro- and anti-inflammatory factors may contribute to carcinogenesis- further research will be required to identify the context for each.

Biological Plausibility

Biological Plausibility is Moderate. Tissue environment is known to be a major factor in carcinogenesis, and inflammatory processes are implicated in the development and invasiveness of breast and other cancers. Studies suggest carcinogenic effects of IR extend beyond DNA damage and mutation of directly affected cells (Bouchard, Bouvette et al. 2013; Sidharan, Asaithamby et al. 2015; Barcellos-Hoff and Mao 2016), including indirect effects through exposed stroma of mammary gland (Nguyen, Oketch-Rabah et al. 2011; Nguyen, Fredlund et al. 2013; Ila-Bochaca, Ouyang et al. 2014). Inflammatory reactions offer one possible mechanism. Tumors and tumor cells exhibit features of inflammation, and inflammation is generally understood to promote transformation and tumor progression by supporting multiple hallmarks of cancer including oxidative activity and DNA damage, survival and proliferation, angiogenesis, and invasion and metastasis (Iliopoulos, Hirsch et al. 2009; Hanahan and Weinberg 2011; Esquivel-Velazquez, Ostoa-Saloma et al. 2015).

In photocarcinogenesis, cytokines and inflammatory signaling are implicated in immunosuppression and in promoting DNA damage via RONS (Valejo Coelho, Matos et al. 2016). In addition, inflammation related NF-kB, STAT3, COX2 and prostaglandins are implicated in the development and proliferation of skin cancers (Martens, Seebode et al. 2018).

Multiple cytokines and inflammatory pathways are implicated in mammary tumors and breast cancer. Cytokines TGF-b and IL6 transform primary human mammospheres and pre-malignant mammary epithelial cell lines in vitro and make them tumorigenic in vivo (Sansone, Storci et al. 2007; Iliopoulos, Hirsch et al. 2009; Nguyen, Oketch-Rabah et al. 2011). IL6 is expressed by breast cancer fibroblasts and by fibroblasts from common sites of breast metastasis (breast, lung, and bone). IL6 is required for the growth and tumor promoting effects of fibroblasts from these sites on ER-positive (MCF-7) cancer cells in vitro and in vivo. IL6 can also promote the expression of IL6 in senescent (skin) fibroblasts and pre-malignant ER- breast epithelial cells (MCF10A). (Sasser, Sullivan et al. 2007; Studebaker, Storci et al. 2008). The growth and invasion-promoting effects of IL6 on primary non-cancer and cancer cell line (MCF-7) mammospheres in vitro depends on the activity of transcription factor NOTCH3, which supports the renewal of stem-like cell populations (Sansone, Storci et al. 2007). The inflammation-related transcription factor NF-kB contributes to mammary tumorigenesis and metastasis in PyVt mice (Connelly, Barham et al. 2011), and NF-kB/IL6/STAT3 activation is essential to mammosphere formation and migration in vitro as well as tumorigenesis from Src-activated or IL6 transformed MCF10 cells (Iliopoulos, Hirsch et al. 2009). The NF-kB/IL6/STAT3 signaling pathway generates cancer stem cells in multiple types of breast cancer cell lines and primary cancer cells (Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Iliopoulos, Hirsch et al. 2011) and is also implicated in colon and other cancers (Iliopoulos, Jaeger et al. 2010).

Empirical Evidence

Empirical Evidence is Moderate. Interventions to increase inflammatory factors increase the carcinogenic potential of targeted and non-targeted cells. Inflammation is documented at earlier time points than tumorigenesis or invasion- within minutes or hours compared to days to months for carcinogenesis, consistent with an inflammatory mechanism of tumorigenesis and invasion. Inhibition of cytokines, inflammatory signaling pathways, and downstream effectors of inflammation activity prevent transformation, tumorigenesis, and invasion (including EMT and senescence) following IR or stimulation of inflammatory pathways. However, the key event and the adverse

outcome differ in their dose-response to ionizing radiation: inflammation does not increase linearly with dose, while breast cancer and invasion does. Uncertainty arises from differences between the CBA/Ca mouse susceptible to leukemia from IR and the BALB/c mouse susceptible to mammary tumors from IR- the former has a pro-inflammatory response while the latter is apparently a mix of anti- and pro-inflammatory. This is a reminder that both pro- and anti-inflammatory factors may contribute to carcinogenesis- further research will be required to identify the context for each.

Interventions to increase inflammatory factors in vitro increase the carcinogenic potential of targeted and non-targeted cells. Stimulating inflammatory pathways with TNF- α , TGF- β , or IL6 increases transformation of pre-malignant embryonic fibroblast and breast epithelial cell lines (MCF10, CDBGeo) cells in vitro (Yan, Wang et al. 2006; Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Nguyen, Oketch-Rabah et al. 2011) and TGF- β and IL6 promote tumor formation by transplanted pre-malignant (MCF10A) and cancer cell lines (MCF-7, SRC-transformed MCF10A) (Sasser, Sullivan et al. 2007; Studebaker, Storci et al. 2008; Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Iliopoulos, Hirsch et al. 2011; Nguyen, Oketch-Rabah et al. 2011). In addition, TGF- β and IL6 promote senescence in fibroblasts (Studebaker, Storci et al. 2008; Liakou, Mavrogenatou et al. 2016) and TGF- β promotes EMT in epithelial cells (Park, Henshall-Powell et al. 2003; Andarawewa, Costes et al. 2011; Iizuka, Sasatani et al. 2017), and senescent fibroblasts and EMT cells support invasion of non-cancer and cancer cells in vitro (Kim, Kim et al. 2004; Andarawewa, Erickson et al. 2007; Sansone, Storci et al. 2007). Most studies used a single dose, but the effects of IL6 on the growth rate of MCF7 cells and of TGF- β on migration and invasion of MCF10A cells are dose-dependent (Kim, Kim et al. 2004; Sasser, Sullivan et al. 2007).

In animal models of carcinogenesis, inflammation is documented at earlier time points than tumorigenesis or invasion- within minutes or hours compared to days to months for carcinogenesis. This temporal concordance is consistent with an inflammatory mechanism of tumorigenesis and invasion. NF- κ B activation increases with time and prior to the appearance of mammary tumors in a mouse model of mammary gland tumors (PyVT) (Connelly, Barham et al. 2011). IR increases both inflammatory signaling and tumorigenesis or invasion. Although only one study (Bouchard, Bouvette et al. 2013) measures both key event and adverse outcome in the same experiment, several studies report that inflammatory signals are enriched in IR-induced mammary gland cancers (Nguyen, Oketch-Rabah et al. 2011; Nguyen, Fredlund et al. 2013; Illa-Bochaca, Ouyang et al. 2014), and polymorphisms in inflammatory genes influence susceptibility to intestinal cancer following IR (Elahi, Suraweera et al. 2009).

Inflammation and carcinogenesis do not have the same dose-response to ionizing radiation: inflammatory signals do not always increase linearly with dose, while carcinogenesis does. To our knowledge no studies examine both early inflammation and tumorigenesis or invasion in response to multiple doses of ionizing radiation, so we instead compare the responses reported separately. TGF- β increases with dose in mammary gland (Ehrhart, Segarini et al. 1997) and IL6 and IL8 increase with IR dose in endothelial cells, but other cytokines (IL1, TNF- α) do not (Meeren, Bertho et al. 1997; Natarajan, Gibbons et al. 2007). Studies in cardiac cells and monocytes report a mixture of linear and non-monotonic dose response (El-Saghire, Thierens et al. 2013; Monceau, Meziani et al. 2013), and one study with fractionated low dose (repeated doses of <0.00003 Gy each, totaling 0.06-0.16 Gy) reported an inverse relationship between dose and inflammation (Ebrahimi, Beugnies et al. 2018). In contrast, carcinogenesis increases with IR dose (Tsal, Chuang et al. 2005; Nguyen, Oketch-Rabah et al. 2011). This discrepancy in dose response suggests that for IR, inflammation is not likely to be the sole factor driving carcinogenesis.

Inhibition of cytokines, inflammatory signaling pathways, and downstream effectors of inflammation activity prevent transformation, tumorigenesis, and invasion (including EMT and senescence) following IR or stimulation of inflammatory pathways. Targeted inhibition of NF- κ B activation in mammary epithelium increases tumor latency and decreases tumor burden and metastasis in the PyVT mouse model of mammary carcinogenesis (Connelly, Barham et al. 2011). Inhibition of COX2, IL6, or addition of antioxidants reduces transformation by IR, IL6-expressing fibroblasts, or TNF- α (Bisht, Bradbury et al. 2003; Yan, Wang et al. 2006; Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Iliopoulos, Hirsch et al. 2011), and inhibition of IL6 reduces tumor formation by SRC oncogene, IL6-transformed or stimulated cancer cells, and IL6 expressing fibroblasts (Sasser, Sullivan et al. 2007; Studebaker, Storci et al. 2008; Iliopoulos, Hirsch et al. 2009; Iliopoulos, Jaeger et al. 2010; Iliopoulos, Hirsch et al. 2011). Although inhibiting IL6 could not reduce senescence induced by IR in human fetal lung and neonatal foreskin fibroblasts and measured by senescence-associated β -galactosidase (Perrott, Wiley et al. 2017), TGF- β and SMAD inhibitors reduced expression of senescence marker SDC1 induced by IR (Liakou, Mavrogenatou et al. 2016). Antibodies to TGF- β block EMT and invasion induced by IR, and MAPK, MMP, and ERK inhibitors reduce the EMT and mobility or invasion induced by TGF- β (Kim, Kim et al. 2004; Andarawewa, Erickson et al. 2007). Inhibiting NOTCH prevents the invasion of MCF7 cells treated with IL6 (Sansone, Storci et al. 2007).

Most (around 85%) of the evidence linking inflammation with tumorigenesis and invasion is from mammary gland or mammary fibroblasts or epithelial cells. Evidence for inflammation following IR, however, is from a wide range of tissue including endothelia, heart, lung, and includes two studies in mammary gland documenting elevated TGF- β , IL6, and COX2.

Uncertainties and Inconsistencies

Uncertainty arises from the multifunctional nature of TGF- β , which may be anti- or pro-carcinogenic based on context, and around the contribution of inflammatory macrophages, which can differ based on genetic background. Further research is needed to isolate and identify the critical factors in these responses and their application in mammary gland.

TGF- β can be protective in a developmental context but may increase risk in another context. Increased baseline TGF- β decreases tumor incidence following lower doses of IR (0.1 Gy) in the SPRET outbred mouse, possibly by reducing ductal branching during development and subsequent susceptibility (Zhang, Lo et al. 2015). Conversely, the BALB/c mouse has lower baseline TGF- β during development but is susceptible to mammary tumors after IR, possibly via an elevated TGF- β response to IR. Early (4 hours) after low dose (0.075 Gy) IR these mice have suppressed immune pathways and macrophage response but increased IL6, COX2, and TGF- β pathway activation in mammary gland compared to the tumor-resistant C57BL/6 mouse (Snijders, Marchetti et al. 2012; Bouchard, Bouvette et al. 2013). By 1 week after IR BALB/c mammary glands show TGF- β -dependent inflammation, and by 1 month after IR they show proliferation (Nguyen, Martinez-Ruiz et al. 2011; Snijders, Marchetti et al. 2012). Consistent with this pattern, BALB/c mice that are heterozygous for TGF- β are more resistant to mammary tumorigenesis following IR (Nguyen, Oketch-Rabah et al. 2011). This pattern suggests that TGF- β is associated with inflammation, proliferation, and mammary tumorigenesis in these mice. However, the BALB/c mouse also has a polymorphism in a DNA repair gene associated with IR-induced genomic instability (Yu, Okayasu et al. 2001), making it difficult to distinguish potentially overlapping mechanisms.

Genetically susceptible mouse models offer somewhat conflicting information about the contribution of inflammation to cancer. In the CBA/Ca mouse susceptible to leukemia the macrophage response to IR is pro-inflammatory (M1 type) in contrast to the mammary tumor resistant C57BL/6 mouse, which develops anti-inflammatory M2type pro-phagocytic oxidative macrophages that target apoptotic cells (Lorimore, Coates et al. 2001; Lorimore, Chrystal et al. 2008). In contrast, in the BALB/c mouse susceptible to mammary tumors many inflammatory pathways and macrophages are suppressed early after IR, although there is also evidence of inflammation especially at later points (Nguyen, Martinez-Ruiz et al. 2011; Snijders, Marchetti et al. 2012; Bouchard, Bouvette et al. 2013). It is possible that the two carcinogenic models represent two different mechanisms of susceptibility.

Finally, inflammation and other stromal factors alone are not sufficient to produce breast cancer. Studies in mice that support the importance of stromal context to IR tumorigenesis used epithelial cells with mutations in a DNA damage response gene p53. These transplant studies irradiate a mammary gland fat pad with epithelial cells removed, and transplant non-irradiated mutant (typically p53 mutant) epithelial cells (Barcellos-Hoff and Ravani 2000; Nguyen, Oketch-Rabah et al. 2011). Similar experiments showing NMLU-treated stromal promotion of tumorigenesis use untreated primary epithelial cells sub-cultured repeatedly in vitro where some initiation could have taken place (Maffini, Soto et al. 2004), while in a similar experiment DMBA-treated stroma does not cause tumors from transplanted pre-malignant immortal cells (Medina and Kittrell 2005). This dependence on both stromal context and mutations to DNA damage response is consistent with contemporary ideas about the multi-factorial nature of carcinogenesis.

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