THE RELATIONSHIP BETWEEN WNT (WINGLESS/INT) PATHWAYS REACTIVE OXYGEN SPECIES (ROS) AND CANCER

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ABSTRACT

Reactive oxygen species has been known to play a role in the formation of cancer and determination of cellular death. Less known, however, is its more recently studied role in regulating cellular physiologic functions. The mechanism of how ROS switches between these roles involves detailed interactions with different pathways. The Wnt pathway, specifically the canonical Wnt/β-catenin pathway, seems to be one that has this role switching effect, depending on the amount of ROS present in the cellular microenvirent. This review aims to revisit the regulation of ROS levels, their relationship to cancer, and how the Wnt pathways influence the effect of ROS.

Key Words : *ROS, Wnt, β-catenin, cancer, nucleoredoxin (Nrx), TCF/LEF, Fox.*

INTRODUCTION

Reactive oxygen species (ROS) are chemically reactive oxygen-containing molecules that play a crucial role in the biology of living organisms. It has previously been shown that excessive levels of ROS can cause cellular damage, as well as lead to ageing and cancer (Serrano & Blasco, 2007). However, recent studies have recognized their role as physiologic signalling molecules that drive cell function when maintained at a balanced low level, and their dysregulation contributing to various pathologies (Katsuyama, Matsuno, & Yabe-Nishimura, 2012; Zhou, Shao, & Spitz, 2014). One group of molecules that seem to have significant effect on this balance is the diverse family of the Wnt signalling glycoproteins (R. Nusse & Clevers, 2017).

It has been proposed that one of the three known Wnt signalling pathways, the Wnt/β-catenin pathway, plays a role in directing cell response to oxidative stress. Under normal physiological conditions, Wnt ligands stimulates a cascade of signalling events that regulate cellular proliferation with a unique feature of being responsible for directional instructions essential for growth and development (R. Nusse & Clevers, 2017). β-catenin nuclear localization is a result of canonical Wnt signalling in the 'on' state, where it acts as a cofactor for the transcriptional activity of T-cell factor (TCF) and Lymphoid enhancer-binding factor (LEF). However, recent evidence suggest that as well as its traditional role as a downstream effector, β-catenin may also bind to several other transcription molecules, for example the O-class of Forkhead box (FoxO) transcription factors, whose target genes are mainly related to the cellular responseof oxidative stress(Hoogeboom &

Burgering, 2009). This occurred in the presence of ROS, suggesting the pivotal role of the Wnt/β-catenin pathway in determining the cell response to oxidative stress.

Reactive Oxygen Species

ROS regulation: generation and elimination

ROS are formed as the result of aerobic metabolism, for example in the mitochondria through the electron transport chain, when O_2 is not fully reduced to water. This partial reduction may result in free radical molecules, such as O_2^- (superoxide), which is readily converted into other forms of ROS, including $^{\bullet}HO$ (hydroxyl radicals);these moleculeshave unpaired electrons and will oxidize other molecules, resulting in a cascade of instability(Lam, Huang, & Brumell, 2010; Pande et al., 2015). Non-radical ROS may also be produced, for example H_2O_2 (hydrogen peroxide), where all electrons are paired, but the molecule is still not in a preferred stable state, and thus can easily transform into hydroxyl radicalsthrough the Fenton reaction or into HOCl by the enzyme myeloperoxidase, although it may also be fully reduced into water by catalase(Lam et al., 2010). In addition, ROS may also be produced by the transfer of electrons from NADPH to O_2 when the former is oxidized by the NADPH oxidase (NOX) enzymes located on cell membranes.

ROS generation by NOXs was originally discovered as a mechanism of cellular defence where NOX-derived ROS would kill pathogens engulfed by phagocytes(Lam et al., 2010). It is now understood that ROS generated by NOX also mediates signalling in numerous cell types, where it is proposed to also play a role in facilitating the survival of cancer cells (Zhou et al., 2014). Other than these two main sources, ROS may also be generated from several other sources, including peroxisomes (Antonenkov, Grunau, Ohlmeier, & Hiltunen, 2010), metabolic activity of cytochrome P450 (Prasad, Mah, Lewis, & Plettner, 2013), and immune cells in chronic inflammation (Nathan & Cunningham-Bussel, 2013).

To ensure the maintenance of low level ROS in normal cells and theprotectionof the deteriorative effects of high levels of ROS, ROS generation is compensated by elimination pathways, which rely on dietary antioxidants, such as vitamins C and E, reducing-molecules, and antioxidant enzymes(Zhou et al., 2014). Superoxide dismutase (SOD) is an antioxidant enzyme that converts the highly unstable superoxide into the non-radical ROS, H_2O_2. Three classes have been characterized: the copper-zinc SOD1 (CuZnSOD) found in cytosol, as well as

the outer mothochondrial membrane (OMM), manganese-containing SOD2 (MnSOD)in mitochondria, and the extracellular SOD3 (ecSOD) which also contains copper and zinc (Che, Wang, Li, Wang, & Zheng, 2016). Catalase is another antioxidant enzyme, contained within peroxisomes, which reduces H_2O_2 into water and O_2(Zhou et al., 2014).

The reducing molecule glutathione is involved in another mechanism of ROS elimination where its reduced form (GSH) can be oxidized at its cysteine residue by ROS to form GSSG (two glutathione molecules adjoined by a disulphide bond) (Zhou et al., 2014). Thioredoxin (Trx) is another reducing molecule crucial for redox regulation, where Trx1 can be found in the cytosol while Trx2 in the mitochondria (Lee, Kim, & Lee, 2013). Nucleoredoxin, a member of the thioredoxin family originally found in the nucleus, was reported as a regulator of the Wnt/β-catenin pathway (Funato, Michiue, Asashima, & Miki, 2006).

ROS Regulation and Cancer

Studies have suggested the dual role of ROS in cancer formation and progression, where it promotes cell transformation towards a cancerous nature on one hand; while on the other hand, it may also trigger cancer cell death(Reczek & Chandel, 2017). Cancer cells have an altered metabolic state where they not only produce more ROS, but also adapt to it by up-regulating their antioxidant systems, resulting in overactive cellular signalling, driving the cell survival and proliferation characteristics of cancerous cells (Moloney & Cotter, 2017). For example, although a decrease of MnSOD activity was consistently found in the initial stages of many cancerous cells, recent evidence suggest that this may not depict the whole picture, as MnSOD is found to be up-regulated as the cancer develops (Dhar & St Clair, 2012). Similarly, during cancer progression, catalase has been found to be responsible for cancer cell resistance to apoptosis through the HOCl pathway, which is dependent on H_2O_2(Bauer, 2012; Scheit & Bauer, 2015). This shows the complex and stage-dependent regulation of carcinogenesis by ROS, where, the over accumulation of ROS, that would have caused cell death in normal cells, is neutralized by the parallel increase in ROS scavenging.

Wnt Signalling Pathways

Over 40 years ago, the wingless gene was reported to regulate the development pattern of *Drosophila melanogaster* (Zhan, Rindtorff, & Boutros, 2017). R. Nusse and Varmus (1982) then discovered the mammalian homolog, Int-1, as an oncogenic provirus

found in mouse mammary tumours. The nomenclature was later adjusted as the Wnt (Wingless/Int) family where Int-1 became Wnt-1 (R Nusse et al., 1991). Three Wnt signalling pathways have been characterised, the β-catenin-dependent canonical Wnt signalling, and the two non-canonical pathways, planar cell polarity (PCP) and calcium-dependent signalling, all of which are initiated by the interaction between the Wnt ligand and receptor (Zhan et al., 2017).

Wnt signalling is highly conserved across species and is one of the pathways involved in the development stages of organisms with the speciality of giving a direction for the growth (R. Nusse & Clevers, 2017). It also maintains the properties of stem cells in adult tissues(Zhan et al., 2017). Correlations between aberrant Wnt signallingand many types of cancer has thus been observed (Clements et al., 2002; Dahmen et al., 2001; Nishisho et al., 1991; Pohl et al., 2017; Satoh et al., 2000; Segditsas & Tomlinson, 2006).

Wnt/β-Catenin Pathway

The canonical Wnt pathway regulates the level of cytosolic β-catenin, where in the absence of a Wnt signal (the "off" state), the β-catenin destruction complex is intact, resulting in a low level of β-catenin. The β-catenin destruction complex is comprised of Axin (which acts as a scaffold protein), adenomatous polyposis coli (APC; whose mutation is related to colorectal cancer), GSK3β (which phosphorylates β-catenin), CK1α, and βTrCP,which ubiquitinatesβ-catenin, taggingit for degradation by proteasome (Latres, Chiaur, & Pagano, 1999). In contrast, when a Wnt ligand binds to the receptor complex (the "on" state), the destruction complex is disrupted, resulting in a stabilized high level of cytosolic β-catenin, which leads to an increased nuclear localization (Zhan et al., 2017). In the nucleus, β-catenin acts as a cofactor for the transcription factors TCF and LEF, replacing the repressive complex of transducing-like enhancer (TLE/Groucho) protein (MacDonald, Tamai, & He, 2009). This results in the transcription of the Wnt target genes and the initiation of various cellular processes (Clevers, 2006; MacDonald et al., 2009).

The signalling is activated when a Wnt ligand binds to the receptor complex of Frizzled (Fzd) receptor and Lipoprotein receptor-related protein-5 (LRP-5) or LRP-6 co-receptor. Glycogen synthase kinase-3β (GSK3β) and casein kinase-1α (CK1α) are then recruited and phosphorylate LRP-5/6 which then recruits Dishevelled (Dvl) proteins to the plasma membrane, where they become polymerized and active (Metcalfe, Mendoza-Topaz, Mieszczanek, & Bienz, 2010). Dvl then inactivates the β-catenin

destruction complex, thus allowing β-catenin accumulation in the cytosol and translocation to the nucleus.

Planar Cell Polarity Pathway

The planar cell polarity (PCP) pathway is essential for the organization of tissues by coordinating the polarity of adjacent cells in a two dimensional sheet of cells amongst other cellular processes related to the orientation of cellular growth (Li et al., 2011).Similar to the canonical Wnt signalling, non-canonical Wnt ligandsbind to the receptor complex of Fzd receptor and recruit Dvl to the plasma membrane (De Marco et al., 2014). Research by Qi et al. (2017) suggests that Dvl, particularly its C terminus, controls the activation of both pathways. In the PCP pathway, through interaction with Dvl-associated activator of morphogenesis 1 (Daam1), Dvl then binds to Rho, leading to its activation and Rho-associated protein kinase (ROCK) mediated signalling, and cytoskeleton rearrangement (Pataki, Couchman, & Brabek, 2015). Dvl also binds to Rac 1 and activate c-Jun-N-terminal kinase (JNK) leading to the transcription of PCP pathway target genes(Pataki et al., 2015).

Wnt/Calcium Pathway

In Wnt/Ca^{2+}signalling, the binding of a Wnt ligand leads to the activation of phospholipase C, followed by an influx of Ca^{2+}into the cytoplasm(Wang, 2009). This then activates downstream effectors including protein kinase C (PKC) and and Cam kinase (Ca^{2+}/calmodulin-dependent kinase (CamK) (Kikuchi, Yamamoto, Sato, & Matsumoto, 2012). This results in many cellular effects, including inhibition of Wnt/β-catenin signalling, alteration to cell adhesion, and cell differentiation (Miller & McCrea, 2010). New modulators of the pathway are gradually being recognized, for example the Calpain2 protein that is activated by Wnt5a, which takes part in regulating mesoderm migration and mesoderm and neural convergent extension (Zanardelli, Christodoulou, & Skourides, 2013). It is also thought to modulate the crosstalk between the Wnt/Ca^{2+} and Wnt/PCP pathways (Zanardelli et al., 2013).

ROS-Wnt Pathway Interaction

ROS Enhances Wnt/β-Catenin Signalling Through Nrx

Nucleoredoxin (Nrx), is a member of the thioredoxin family and is predominately found in the nucleus. Funato*et al*. identified it as a regulator of Wnt signalling for its role in disrupting the β-catenin cytoplasmic destruction complex (Funato et al., 2006). In this study, they found that Nrx interacts with cytoplasmic Dvl, preventing its recruitment to the plasma membrane via interaction with the cytoplasmic portion

of the transmembrane Frizzled receptor. This Nrx-bound Dvl cannot be recruited and disruption the β-catenin destruction complex occurs, thus leading to the eventual degradation of β-catenin. Furthermore, Funato *et al*. (2006)demonstrated that this Nrx-Dvl binding is interrupted by ROS, in this case H_2O_2, stabilizing the cytoplasmic β-catenin concentration, and increasing downstream TCF/LEF transcription factor activity. They concluded that ROS had positive effects on Wnt/β-catenin signalling. In addition to this, a subsequent study by Funato *et al*. (2010)reported that the binding of Nrx to Dvl not only prevents Dvl activation in the Wnt/β-catenin pathway, but also prevented it from being degraded by Kelch-like 12 (KLHL 12). These studies highlight the importance of the antioxidant molecule, Nrx, in the ROS-mediated regulation of Wnt/β-catenin signalling.

Kajla *et al*. (2012)conducted a study that further enhances the knowledge on this matter, where they found that Wnt ligand-receptor interaction itself induces ROS production by NADPH oxidase 1 (Nox1) that causes the Nrx-Dvl dissociation. This process happens initially through Wnt activation of Src (a tyrosine kinase), followed by Src phosphorylation of Vav2 (a Rac guanine-nucleotide exchange factor), which in turn induces the activation of Rac1 (a GTPase from the Rho superfamily) through the switch of bound molecule GDP (released) to GTP (captured) (Kajla et al., 2012). Rac1 then transduces the signal to NOX-1, causing ROS production in the form of superoxide, which is both time and dose dependent(Kajla et al., 2012). Nox1-derived ROS oxidises Nrx, causing its disassociation from Dvl, which eventuates with the disruption of β-catenin destruction complex and activation of the Wnt/β-catenin signalling pathway (Kajla et al., 2012).

Consistent with the findings of Funato*et al*., Kim *et al*. have also demonstrated ROS-induced activation of the Wnt/β-catenin pathway (Kim, Kim, Na, & Surh, 2010). Human breast epithelial cells, from the MCF-10A line, treated with tumour necrosis factor-α (TNF-α, also referred to as TNF, a pro-inflammatory cytokine believed to be the link between inflammation and tumour formation) resulted in an increased expression of urokinase-type plasminogen activator (uPA, a serine protease involved in extracellular matrix degradation in cancer) by increasing nuclear localization of β-catenin and increasing TCF-4 binding to DNA, both indicating activation of Wnt signalling, which was mediated by an increase of intracellular ROS, possibly through the mechanism

described by Funato et al (Kim et al., 2010).

In contrast with these findings, studies have reported results where treatment with H_2O_2 causes a decreased Wnt/β-catenin signalling, mainly due to the dual roles of β-catenin, where it binds to and increases the transcription activity of not only TCF/LEF transcription factors, but also Forkhead box-O (FoxO), in which β-catenin seems to have binding preferences with the latter, resulting in shift from the traditional TCF/LEF transcription activity (Almeida, Han, Martin-Millan, O'Brien, & Manolagas, 2007; Dong et al., 2013; Essers et al., 2005; Hoogeboom et al., 2008). Another mechanism reported to lead to the negative effects of ROS on Wnt/β-catenin signalling pathway is the direct downregulation of β-catenin by ROS by activation of GSK3β(Shin, Chin, Lee, & Kim, 2006; Shin et al., 2004).

ROS Decreases Wnt Target Gene Expressions Due To The Diversion Of B-Catenin Binding, From TCF/LEF to FoxO Transcription Factors

FOXO is known to regulate the expression of genes required for cellular response to oxidative stress such as catalase and MnSOD (Hoogeboom & Burgering, 2009). However, their activity, in turn, is regulated by ROS. This regulation was reported by Essers *et al*. (2005) from studies conducted on various cell lines and *in vivo* on *Caenorhabditiselegans*, where they demonstrated the binding of β-catenin to FOXO, which is found to be enhanced by H_2O_2stimulation. Reinforcing these results, Almeida *et al*. (2007),studied mesenchymal and osteoblastic cell lines and reported an interactionbetween FOXO and β-catenin which was enhanced by H_2O_2, followed by an increase in FOXO transcriptional activity. On the other hand, contrary to the effects on FOXO, the transcriptional activity of TCF is supressed in the presence of an increasing dose of H_2O_2 and that this effect occurs downstream of β-catenin(Almeida et al., 2007). Furthermore, they also reported that this suppression of TCF activity in the presence of H2O2 was rescued by overexpressing β-catenin, suggesting that this suppressive effect is due to a diversion of β-catenin binding preference from TCF to FOXO within a limited pool of β-catenin(Almeida et al., 2007). Similar results of this competitive nature between TCF and FoxO transcription factors were reported by Hoogeboom et al. (2008) from experiments on DL23 and DLD1 human colon carcinoma cells.

In line with the previous studies, Dong et al. (2013) showed thatfructose 1,6-biphosphatase (FBP1)increases ROS production, and decreased the interaction

between β-catenin and TCF, while increasing its interaction with FoxO. This then resulted in a suppression of basal-like breast cancer (BLBC) stem cells and inhibited the tumour from enhancing. Furthermore, McClelland Descalzo et al. (2016) showed that glucose induces ROS production in embryonic stem cells and activation of FoxO transcription factor, which not only increases ROS scavenging through the increased expression of the antioxidant FoxO target genes, but also decreases the proliferation of the cells.

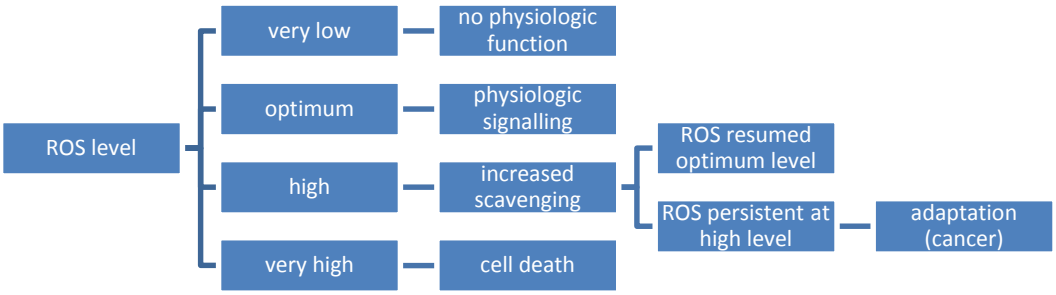

Figure 1*: ROS level and possible outcomes*

CONCLUSION

It has been established that ROS is needed as signalling molecules for physiologic functions. However, it is very crucial to maintain the ROS homeostasis (Figure 1). Levels that are below the physiologic threshold will impede physiological signalling, while levels that are above the threshold appears to manifest in three ways: activation of antioxidant genes (FoxO targets) that will result in increased ROS scavenging and resume of the balance; activation of antioxidant genes that could not resolve the issue but lead to the cellular adaptation to the high levels of ROS instead, a phenomenon observed in cancer cells; and activation of ROS-induced cell death. The Wnt/β-catenin pathway seems to play an intricate role in maintaining this balance, through the effects of ROS on Dishevelled, GSK3β, and β-catenin transcription complexes. Other than the type of cell and level of ROS itself, the duration of ROS exposure as well as the source of increased ROS seems to have effect on how the Wnt pathway is modulated in the presence of ROS. Much research is still needed to understand the complex interactions.

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