P53: A Mediator of Life and Death

How possible interactions of p53 with the HIF-1, Nrf2 and ATF4 pathway can determine cell fate in precision-cut kidney slices.

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Abstract

Precision-cut kidney slices are a promising ex-vivo model for renal toxicological studies. However, as a decrease in viability during incubation was observed in several studies on PCKS, further investigation on the underlying toxic processes is required. Here, the involvement of four transcription-factor-mediated stress response (p53, HIF-1α, Nrf2 and ATF4) is considered. P53 has an important decisive role in determining cell fate. In response to a specific stressor, p53 might interact with the other protective pathways to promote either cell survival or cell death. The results show that specific interactions between p53 and HIF-1α, p53 and Nrf2 and p53 and ATF4 may indeed affect cell fate, depending on the conditions such as nature and duration of the stressor and cell type. Notably, an involvement of MDM2, inhibitor of p53, was found in all three interactions. Therefore, in order to promote cell survival in PCKS during incubation, MDM2 could be a possible target for manipulation.

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1. Introduction

The accelerated development of new drugs over the last decade has created a strong demand for effective and reliable models with which to test the toxicity of future drugs (Dorata & Buckley, 2006). These models should emulate the responses of several organ and tissue systems and ideally help predict the possible harmful effects of the new drug candidates for human subjects (Dorato & Buckley, 2006). Since *in vitro* cell cultures tend to neglect cellular heterogeneity and tissue complexity and since ethical constraints make it almost impossible to obtain the appropriate sample size required for persuasive *in vivo* experiments (Poosti *et al*., 2015), there is a pressing need for an *ex vivo* model that accounts for cell heterogeneity, as provided by precision-cut tissue slices (PCTS).

Since PCTS preserve the multicellular characteristics of the organ, they constitute *ex vivo* models that closely approximate the *in vivo* conditions of a variety of organs, such as the liver, kidney, intestine, brain and spleen (Graaf *et al.,* 2007). Although ex vivo tissue slices were initially used in 1923 by Otto Warburg, the technique did not gain wide acceptance because methods of manual preparation inhibited replicability and viability (Parrish *et al., 1995*). In 1980, Krumdieck developed an automated tool to produce tissue slices of equal and precise thickness (i.e. PCTS, see Krumdieck *et al.,* 1980). Although this instrument improved replicability (Parrish *et al., 1995*), there continued to be issues concerning PCTS viability during incubation. Sustained ATP content suggested that PCTS remained viable and therefore usable for an average of 48-72 hours (Fisher *et al*., 2001). Other studies indicate that viability may vary depending on tissue type. For example, a promising study on human precision-cut liver slices indicates that they remain viable up to five days after incubation (Starokozhko *et al.,* 2016). In contrast, a recently unpublished study on precision-cut kidney slices (PCKS) at the Department of Pharmacokinetics, Toxicology and Targeting at the University of Groningen detected changes in viability at 5, 24 and 48 hours after incubation (Oude Avenhuis, 2016). In view of the high demand for precision-cut kidney slices in toxicological studies on renal function (Kim & Moon, 2012), Oude Avenhuis reveals the need for further study on the mechanism underlying the possible toxic effects of the preparation and incubation process in order to set reliable guidelines on PCKS lifespan.

One well-known source of such toxic effects involves cell response to stress. Even with the introduction of new slicing techniques, preparation and incubation of PCKS undoubtedly causes a certain amount of stress, thus activating the stress response pathways that cells have evolved (Oude Avenhuis, 2016). These pathways are initiated whenever a perturbation in cellular processes occurs, and their primary task is to regain and maintain homeostasis of the cell (Jennings, 2012). Generally, they achieve this end by activating protective responses such as cell repair, temporary adaptation to the stressor or autophagy (Poljša & Milisav, 2012). Ideally, these mechanisms will lead to full recovery and continued survival of the cell, but a mechanism of regulated cell death can be triggered whenever inflicted damage is excessive and cellular homeostasis cannot be obtained. This programmed cell death seemingly contradicts the other three responses, since it is a destructive rather than protective response at the level of the individual cell. However, a mechanism of cellular self-sacrifice may prevent a damaged cell from jeopardizing surrounding tissue due to further differentiation and proliferation. If irreparably damaged cells proliferate and spread throughout a tissue environment, they could cause multiple diseases, including cancer (Meek, 2004). Programmed cell death may be a response that prevents such spreading and the resulting proliferation of damage, once the processes of tissue repair have been exhausted. Both protective and destructive stress response options are therefore essential for tissue viability (Jennings *et al.,* 2012).

Several important conditions contribute to a cell's protective and destructive responses, such as the nature and duration of the stress and the cell type. At the same time, the prevalent issue likely concerns the underlying transcription-factor-mediated molecular pathways, such as NFE2L2 (Nrf2), NFE2L1 (Nrf1), p53, heat shock factor, unfolded protein response (ATF4 and ATF6) and the hypoxia responses (HIF1 and HIF2). These molecular pathways are all regulated by a transcription factor after being exposed to certain types of stress or toxicological insult and induce either a protective or destructive stress response (Jennings *et al.,* 2012). It is therefore important to determine the circumstances that regulate these pathways and to identify the switch (if any) that determines the transition of one to the other type of response in PCKS samples.

Previous research in association with three fellow students examined the diminished viability in mouse PCKS during incubation by investigating the mRNA expression of several genes in four stress response pathways over a timespan of 48 hours. The four selected stress response pathways were (Limonciel *et al*., 2015):

1) Oxidative stress response caused by ischemia and initiated by the Nrf2 pathway

2) Oxidative stress response caused by hypoxia and initiated by the HIF-1 pathway

3) Unfolded protein response (also known as ER stress) activated by the ATF4 pathway

4) DNA damage stress response activated by the p53 pathway

The assumption that these pathways would be expressed in PCKS was justified by the important role that they evidently play in maintaining kidney homeostasis. The HIF-1 pathway, sensitive to changes in levels of blood oxygen, regulates the kidney production of erythropoietin (EPO) (Nangaku & Eckardt, 2007). Studies on the Nrf2 pathway have shown that its absence can encourage acute kidney failure and chronic kidney disease (Shelton *et al.,* 2015). Similarly, ER stress is one of the main causes of renal diseases indicating an important protective role of the ATF4 pathway in kidneys (Taniguchi & Yoshida, 2015). On the other hand, activation of the p53-mediated pathway can have a pathological effect, as has been indicated in studies on cisplatin- and patulin-induced nephrotoxicity (Jiang & Dong, 2008).

All four pathways could possibly contribute to nephrotoxicity, either by up-regulation (p53) or down-regulation (HIF-1, Nrf2 and ATF4). However, even though documented results reveal changes in mRNA expression in each pathway and therefore provide some insight into their contribution to PCKS viability, it is also clear that these pathway are intertwined in a complex network. After all, these interactions might determine the switch between the protective and destructive stress response that could ultimately decide the fate of the stressed cell (Fulda *et al.,* 2010). The Nrf2, the HIF-1a and the ATF4 pathway are all transcription-factor mediated pathways with a protective function that promote cell survival. P53 is a transcription-factor mediated pathway that brings about both cell survival and programmed cell death. Since there are many connections between p53 and the three other pathways, it is quite likely that p53 is able to promote cell survival or programmed cell death by interacting with each pathway separately . In this paper a possible role of p53 as the mediator of cell fate will be discussed. How does the p53 pathway influence the Nrf2, the HIF-1a and the ATF4 pathways and can these interactions be manipulated to prolong PCKS viability?

2. Cell fate and the p53 pathway

The p53-mediated stress response is a collection of cellular responses with different underlying mechanisms positively or negatively regulated by p53. Using a simplified model, the responses can be divided into two categories: a protective response of adaptation and repair, and a destructive response involving programmed cell death (Kruiswijk *et al.,* 2015). Although p53 switch to either cell life or cell death remains a complex topic, the following sections will discuss the progress made in determining the underlying processes.

2.1 P53 in response to stress

The p53 pathway was discovered in 1979 and is widely known for its tumor suppressive function (Jennings *et al.,* 2012). The pathway is activated by certain stressors such as hydrolysis, deamination, oxidative activity and alkylation, all of which can cause DNA damage. Although the response of p53 to such stressors has been well established (Jennings *et al.*, 2012), other types of stress such as hypoxia also activate p53 as a transcriptional factor, which in turn induces p53 response genes. Depending on the stressor, these genes can generate a variety of responses, such as reversible cell-cycle arrest (senescence), differentiation, reversible cell-cycle arrest and apoptosis (figure 1) (Vousden & Lu, 2002). Although p53 can, in some circumstances, protect the cell by activating a DNA repair mechanism, it will, in most cases, function as a destructive mechanism by either activating cell senescence or by inducing apoptosis.

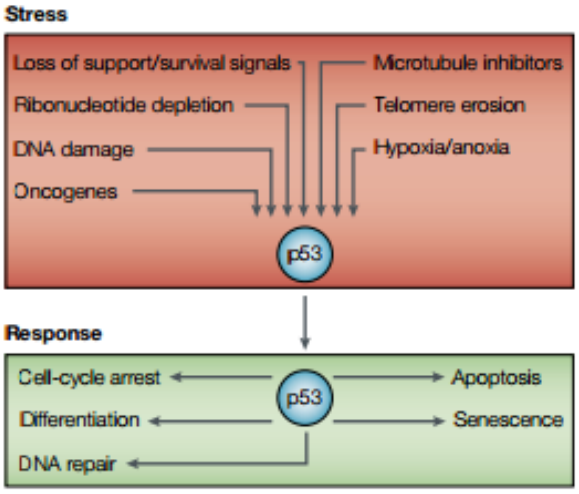

Figure 1: P53 different responses to different types of stressor (Vousden & Lu, 2002)

2.2 Classic model of p53 activation

Due to the tumor suppressive function of p53, it has been subject to extensive study since its discovery (Jennings *et al.,* 2012). A classic p53 mode has been formulated, based on three rate-limiting steps: p53 stabilization (activation phase), sequence-specific DNA binding (effector phase) and target gene activation (outcome phase) (Braithwaite *et al.,* 2006). Although this model remains useful, it is outdated and oversimplified and fails to provide insight into the switch between

protective and destructive responses (see figure 2) (Kruse & Gu, 2009). Here, the classic model will be discussed while incorporating new information from recent findings in order to clarify branches along the pathway and the gateways regulating them.

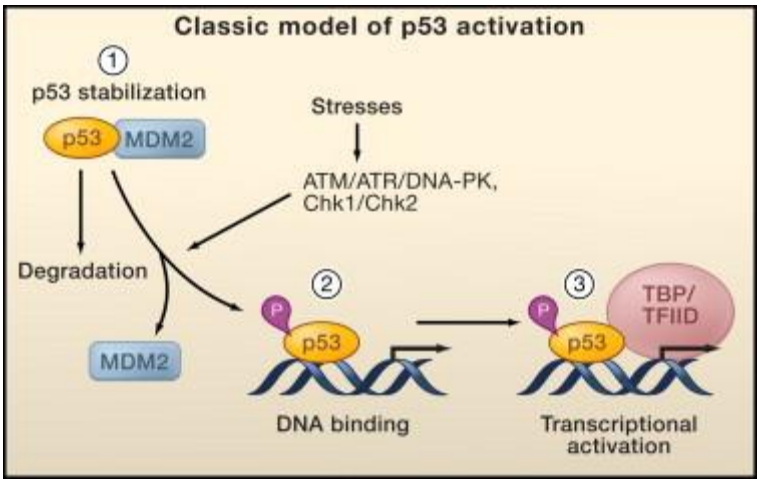

***Figure 2**: Classic model's three steps of p53 activation (*Kruse & Gu, 2009)

2.2.1 Activation phase

Under normal conditions, stable p53 is not found in cytoplasm but comprises a complex with murine double minute 2 and 4 (MDM2, MDM4). MDM2 is an E3 ubiquitin ligase that both blocks p53 function and targets p53 for degradation (Riley *et al.,* 2008). In response to a stress signal, cytoplasmic p53 must first be stabilized and accumulated beyond a threshold concentration before it can induce any activity. In the classic and most-studied model, p53 stabilization occurs through direct inhibition of MDM2 or through posttranslational modifications of p53 that will block its ability to bind with MDM2. For instance, p53 can be modified by phosphorylation by the kinases ATM/ATR/DNA-PK and Chk1/Chk2 (Kruse & Gu, 2009), kinasing phosphorylate serine and threonine residues in the N terminus and resulting in disassociation of MDM2 and p53 (Jennings *et al.,* 2012). Studies on knock-in mice have shown that N-terminal phosphorylation at the Ser15 and Ser20 sequences especially inhibits the binding of MDM2 and p53 (Brooks & Gu, 2010). Despite many *in vitro* and *in vivo* studies on these posttranslational modifications of p53, the exact mechanisms remain unclear (Ljungman, 2000).

2.2.2 Effector phase

The manner in which p53 induces a specific response after activation is subject to debate. The classic model fails to explain the mechanism through which a specific stressor can elicit either a cell protective response or a destructive response. Recent studies indicate that the answer could be found in the posttranslational modifications of p53 after activation (Braithwaite *et al.,* 2006). In a model proposed by Kruse & Gu (2009), histone acetyltransferases (HAT) play an important role in the target-specific binding of p53. Important HATs are co-activators CBP and p300, which form a complex with p53 leading to transactivation of the response genes. If the p53 protein is not acetylated, it will

bind to the promotors of the transcriptional genes for MDM2 and Pirh2 (both negatively regulate p53). If a mild p53 response is desired, HATs will acetylate parts of the transcriptional factor such as the Lys320 residue, after which it can bind to the promotors of genes that will induce cell-cycle arrest and DNA repair (p21 and GADD45) (Carvajal & Manfredi, 2013). Finally, specific modifications such as the acetylation of Lys120 and phosphorylation of Ser46 are required to activate target genes that will result in apoptosis (see figure 3) (Kruse & Gu, 2009) (Carvajal & Manfredi, 2013).

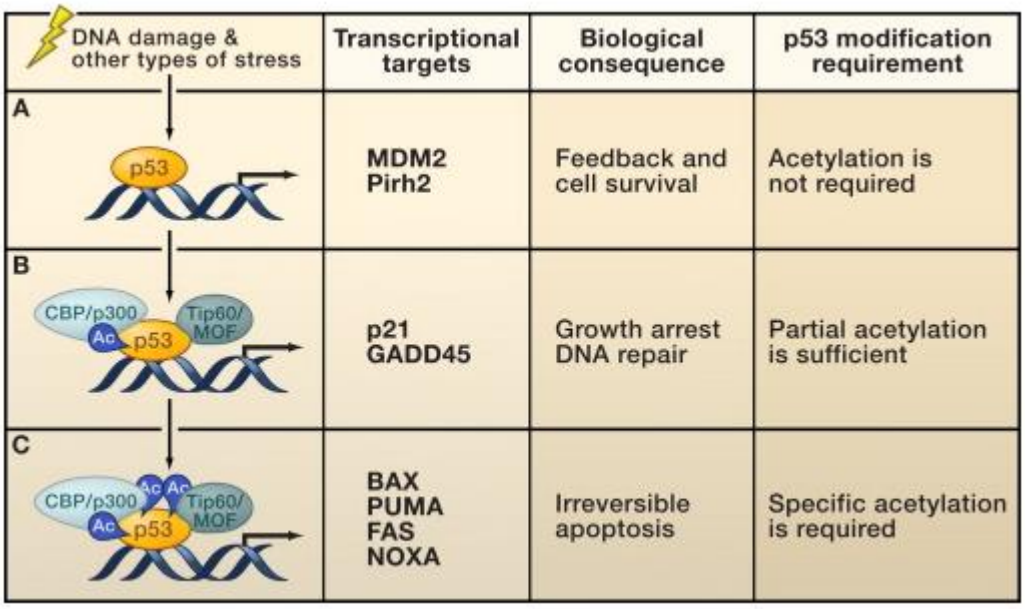

Figure 3: *Influence of posttranslational modifications on different p53 responses (Kruse & Gu, 2009).*

This, however, is not the only hypothesis on the binding specificity of p53. Vousden & Lu (2002) suggest that the binding sites on the promoters of the target genes have different affinities for p53. Their model proposes that the promoter of genes involved in cell-cycle arrest have a higher affinity for p53 than the genes involved in apoptosis. This could explain the many observations that low concentrations of p53 induce a protective cellular response whereas high concentrations induce a destructive response, a hypothesis is encouraged by Carvajal & Manfredi (2013) (figure 4).

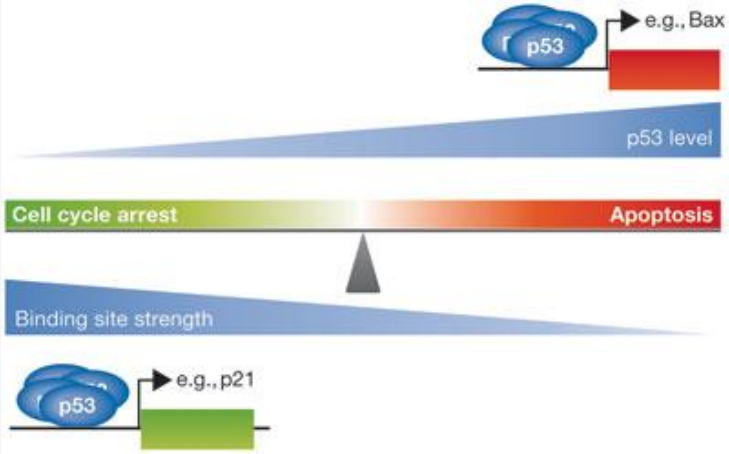

Figure 4: *P53 concentration as determiner of cell fate (Carvajal & Manfredi, 2013)*

2.2.3 Outcome phase

Once p53 selectivity has been set, p53 will bind to the response genes needed to induce the specific outcome.

Apoptosis

The most commonly studied outcome of the p53 pathway is apoptosis, a form of programmed cell death in which cellular components by are degraded by proteolytic enzymes such as caspases (Fulda & Debatin, 2006). Depending on whether the stressor is intra- or extracellular, apoptosis is either induced by the intrinsic (intracellular) or extrinsic (extracellular) apoptotic pathway.

The extrinsic pathway is activated when extracellular death ligands bind with corresponding transmembrane receptors, stimulating caspase 8 and initiating a cascade of downstream effector caspases which will lead to apoptosis (Kruiswijk *et al.,* 2015). Even though the mechanisms are not fully understood, the genes encoding certain ligand receptor families (TRAIL and Fas) seem to be direct targets of p53 (Fridman & Lowe, 2003). In contrast, the p53-controlled intrinsic apoptotic pathway has been extensively studied. The pathway is regulated by the Bcl-2 protein family, which contains both pro-apoptotic factors BAX (Bcl-2 associated X protein) and BAK (Bcl-2 homologous antagonist/killer), as well as anti-apoptotic factors Bcl-2 and Bcl-X. The pro-apoptotic factors can attack the mitochondrial membrane, creating pores through cytochrome *c* is released into the cytoplasm, where it induces apoptosis by activating caspase 9 (Kruiswijk *et al.,* 2015). The anti-apoptotic factors can form a heterodimer with the pro-apoptotic factors, thus inhibiting their function. The crucial ratio between anti- and pro-apoptotic expression is carefully regulated by p53 (Fridman & Lowe, 2003).

Apoptosis is the best known but not only form of p53-induced cell death. There is evidence that p53 also takes part in non-apoptotic cell death (ecroptosis, parthanatos, pyroptosis, autophagy and ferroptosis) (Fridman & Lowe, 2003). Although underlying signaling pathways remain unknown, they could involve non-caspase proteases (Fulda & Debatin, 2006).

Reversible cell-cycle arrest and DNA repair

A slightly less complex and therefore better understood p53-pathway outcome is cell-cycle arrest. It is induced by p53 transactivation of the gene encoding for cyclin-dependent kinase (CDK) inhibitor p21, which inhibits cyclin-dependent kinases involved in the G1 cell-cycle checkpoint, resulting in G1 cell-cycle arrest. P53 can also transactivate GADD45 and 14-3-3σ, which have a similar function to p21 (Braithwaite *et al.,* 2006). If temporary, cell-cycle arrest gives the cell time to repair DNA damage. Indeed, p53 has been recently shown to activate DNA response pathways such as PARP1, which induces DNA repair by assembling histones topoisomerases and DNA helicases (Kruiswijk *et al.,* 2015).

Cellular senescence

Repair mechanism failure will induce a permanent state of cell-cycle arrest (i.e. senescence). The cell still functions but is unable to replicate and therefore proliferate further. Similar to reversible cell-cycle arrest, senescence is believed to be caused by activation of p21, which is then prolonged,

resulting in an overexpression of cyclin-dependent kinase inhibitor $p16^{INK4A}$. However, a study by Kortlever *et al.* (2006) indicates that p53 can also induce senescence by activating expression of plasminogen activator inhibitor 1 (PAI1), which can permanently inhibit an important growth factor pathway (PI3K) (Kortlever *et al.,* 2006).

2.3 The dual role of p53

Although the mechanisms behind the individual processes are slowly coming to light, connections between the different phases of p53 activation and the manner of determining cellular outcome remain unclear. Depending on cell type, nature and duration of stress, p53 concentration and post-translational modifications may induce specific responses (Kruiswijk *et al.,* 2015). However, the simple purpose underlying the complexity of response is to minimize the damage inflicted by the stressor. Under low to mild stress conditions, p53 should function as a protective mechanism in order to repair the cell and maintain homeostasis. Under moderate to high-stress conditions, p53 induces cell destruction, thus preventing the spread of damage.

Presumably, if p53 determines cell fate, it should be able to communicate with other stress response pathways in order to stimulate the desired outcome. Therefore, the following sections will discuss possible interactions of p53 with HIF-1, Nrf2 and ATF4.

3. Determining cell fate under hypoxic conditions

One of the essential conditions for cell survival is oxygen availability. A decrease in oxygen supply (hypoxia) is rapidly detected by the cell, resulting in activation of the hypoxic pathway by transcription factor hypoxia-inducible factor 1 (HIF-1) (Masoud & Li, 2015). HIF-1 reacts to hypoxia by activating the genes involved in glycolysis and hence promoting cellular oxygen production (Bruick *et al.,* 2003). However, severe hypoxia has also been shown to lead to an accumulation of p53, resulting in apoptosis (Hammond *et al.,* 2005). The interaction between p53 and HIF-1 will be analyzed below as the possible switch between cell survival and cell death under hypoxic conditions.

3.1 HIF-1 response to stress

HIF-1 consists of two subunits, HIF-1α and HIF-1β. Only a direct correlation between HIF-1α expression and hypoxia has been found to date (Masoud & Li, 2015). Under normal conditions, the HIF-1α protein has a very short half life (5 minutes). Its ubiquitination and degradation is regulated by von Hippel-Lindau protein (pVHL), one of the components of an E3 ubiquitin ligase complex. To be recognized by pVHL, a group of enzymes called prolyl-4-hydroxylases (PHDs) have to mark HIF-1α by hydroxylation. Being an aerobic process, HIF-1α will not be marked for ubiquitination and degradation by pVHL under hypoxic conditions. HIF-1α can also be activated by hypoxia in a pVHL-independent manner. Under normal conditions, HIF-1*α* asparagine (N^{803}) residue is hydroxylated by factor inhibiting HIF-1 (FIH-1), inhibiting the interaction between HIF-1α and co-activator CBP (CREB-binding protein)/p300 and preventing transactivation of HIF-1α target genes. Hypoxia inhibits FIH-1 activity and therefore promotes gene activation by HIF-1α (Masoud & Li, 2015).

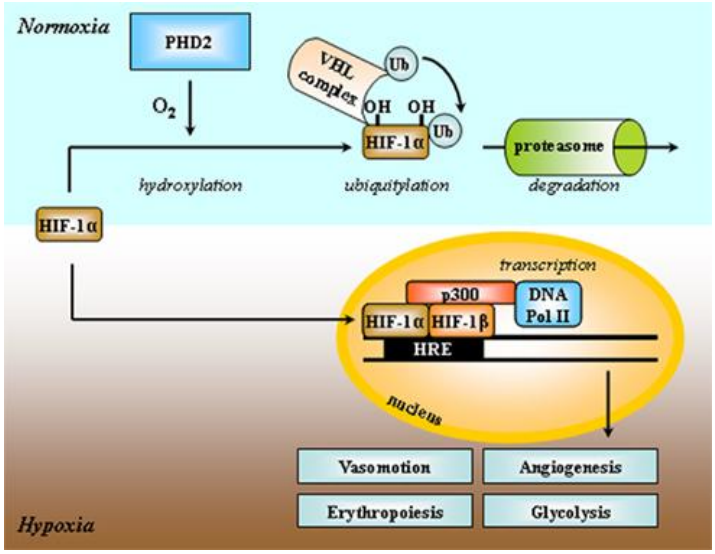

Figure 5: *HIF1 pathway in response to hypoxia (Porporato et al., 2011)*

As hypoxia is a known activator of the p53 pathway, a connection between the HIF-1α pathway and p53 is highly plausible. The following two sections will discuss in the reciprocal influences of HIF-1α and p53.

3.2 Influence of HIF-1α on the p53 pathway

Protein expression

Many studies have indicated that hypoxia leads to a HIF-1α dependent increase in stabilization of p53 protein (Sermeus & Michiels, 2011). Although there is no consensus on the mechanisms behind this process, many investigators have mentioned MDM2-dependent and MDM2-independent processes (Obasz *et al.,* 2013). Chen *et al.* (2003) found that HIF1-α directly binds to MDM2, thus suppressing ubiquitination of p53. No direct connection between p53 and HIF1-α was found, suggesting that MDM2 might act as a mediator between both proteins. One theory about an MDM2-independent connection between p53 and HIF1-α notes that HIF1-α activates kinase ATM, which in turn phosphorylates the serine 15 residue in the N-terminus of p53 (see above)(Brooks & Gu, 2010). Another study proposes that PNUTS, a hypoxia-induced protein, might inhibit a phosphatase for p53 (protein phosphatase-1) and hence stimulate p53 activity (Sermeus & Michiels, 2011).

Many contrasting hypotheses on the mechanism behind p53 stabilization can be found in the literature, but it is widely believed that HIF-1α and MDM2 are involved in the processes (Robertsen *et al.,* 2014).

P53 activity

A study by An *et al.* (1998) reveals increased p53 activity as a result of HIF-1α overexpression under normal conditions. However, experiments have shown that the target genes of p53 are not induced under hypoxia (Sermeus & Michiels, 2011). P53 binds to the promoter without recruiting co-activators CBP and p300. Since these co-activators are required for both the transactivation of HIF-1 and p53, both transcriptional factors might be in competition for the binding domain of p300 (Schmid *et al.,* 2004). This mechanism could be regulated by a target gene for the HIF-1 pathway, cockayne syndrome B (CSB). A study on hypoxic cells with CSB absent revealed high activation of the p53 pathway but a decrease in events downstream of this pathway (Filippi *et al.,* 2008). This suggests that the HIF-1 dependent transcription of CSB negatively influences the binding of p53 to co-activators CBP and p300, leaving more p300 available for HIF-1 transactivation. Another hypothesis for the lack of p53 transactivation suggests that hypoxia might not lead to the specific posttranslational modifications of p53 needed for the recruitment of the co-activators (Sermeus & Michiels, 2011).

As transactivation of p53 is reduced under hypoxic conditions, its main activity is said to be transrepression. It is subsequently claimed that p53 induces apoptosis indirectly by repressing survival enhancing genes. One possible gene targeted by p53 under hypoxic conditions is HIAP/BIRC3, which normally interferes with caspase activation (Sermeus & Michiels, 2011). Another gene that might be repressed in survivin, of which the corresponding protein provides a survival signal for the cell. However, the outcomes of studies on survivin repression differ, suggesting that p53 transrepression of survivin might only occur in certain cell types (Hammond & Giaccia, 2005). According to Sansome *et al.* (2001), HIF1 enhanced activity of p53 could also be DNA-binding-independent. In an experiment on ischemic rats, p53 was found in mitochondria, directly inhibiting the anti-apoptotic gene Bcl2, thereby promoting cell death (Samsone *et al.,* 2001). However, most studies only found p53 activity in the nuclear compartment (Sermeus & Michiels, 2011).

To sum up, most of the studies show a HIF-1 dependent increase in p53 stabilization and activity. However, the response mechanisms are different from the p53 response to other stressors, as p53 is most likely to induce apoptosis through transrepression instead of transactivation of response genes.

3.3 Influence of p53 on the HIF-1α pathway

Protein expression

As HIF-1 can enhance p53 stabilization and activity, a similar inverse relationship may also exist. Indeed, *Ravi et al.*(2000) found that p53 mutant mice (p53 -/-) had enhanced HIF-1α compared to the wildtype (p53 +/+). It seems probable that p53 stimulates degradation of HIF-1α is some still unclear manner. The results of *Ravi et al.*(2000) showed that p53 must be bound to the MDM2 complex in order to have this effect. Others have shown that HIF-1*α* degradation need not require any involvement of MDM2 but may operate by stimulating pVHL. Although the mechanisms remain unclear, most studies have indicated a negative effect of p53 on HIF-1*α* stabilization.

HIF1-α activity

Since HIF1-α and p53 are compete for the binding site of co-factor p300 (Schmid *et al.,* 2004), p53 can affect HIF1-α activity without influencing protein concentration. A number of *in vivo* studies on breast cancer have shown that low p300 concentration combined with high p53 concentration results in strongly decreased HIF1 activity. It is therefore likely that, given limited p300 concentration, high p53 concentration can have a negative effect on HIF1 (Sermeus & Michiels, 2011).

3.4 Balance between p53 and HIF1-α

Although the focus above is on the interaction between p53 and HIF1-α, the pathways do not always affect each other. For example, an article by Halterman & Federoff (1999) note that the stabilization of p53 depended on the severity of the hypoxia condition. In facdt, only HIF-1α is reported to be stabilized at 2% oxygen, whereas both p53 and HIF1-a are stabilized at 0.02% (Sermeus & Michiels, 2011). This suggests that that HIF-1-dependent induction of p53 also depends on the nature and duration of the hypoxia. Moreover, another study by Cosse *et al.* (2007) showed differences in HIF-1α dependent p53 induction between different cell lines, making cell type another variable that needs to be taken into account (Cosse *et al.,* 2007).

Accordingly, a simplified model for the regulation of cell survival and cell death based on the interaction between p53 and HIF-1 has been proposed by Sermeus & Michiels (2011). Under normal conditions, both pathways are only mildly expressed and will not affect the cell. When the cell is exposed to mild hypoxia, high HIF-1α expression will inhibit p53 expression and activity due to co-activator binding competition. Lower concentrations of p53 can stimulate p21-induced cell-cycle arrest (as discussed above) (Carvajal & Manfredi, 2013) so both pathways collaborate in a protective response. However, should the hypoxia last and become more severe, the high expression of HIF-1α will lead to high p53 expression through MDM2 inhibition and induce apoptosis due to transrepression. Finally, high p53 stabilization and activity during anoxia can possibly inhibit HIF-1α expression and activity (see figure 5) (Sermeus & Michiels, 2011).

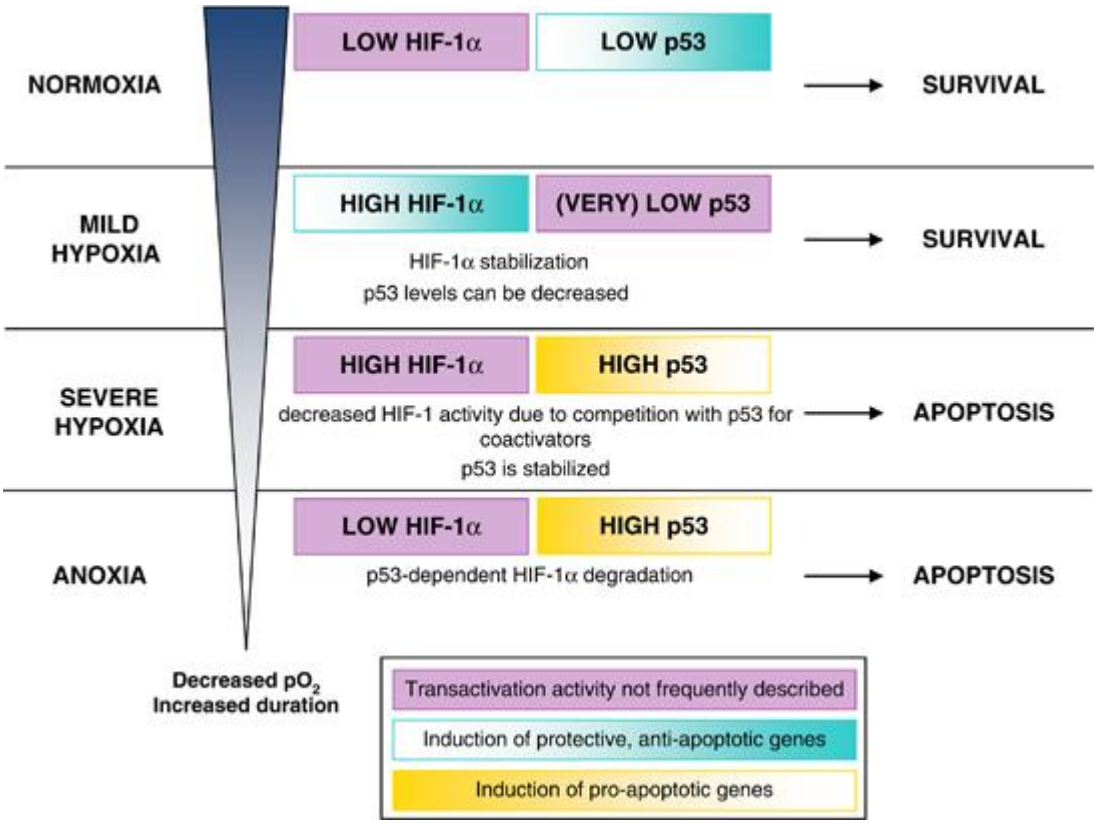

Figure 6: *Proposed interaction between p53 and HIF1 under different hypoxic conditions (Sermeus & Michiels, 2011)*

Although this model remains speculative, it is clear that cell fate is determined by the balance between HIF-1α and p53 pathway under hypoxic conditions. Depending on conditions, the pathways may either collaborate or oppose each other. The crosstalk might yield either a protective or destructive response and therefore remains an important topic for further research.

4. Determining cell fate during oxidative stress

The Nrf2/ARE stress response, also known as the oxidative and electrophilic stress response, is a mechanism to protect the cell against free radicals released, for example, due to inflammation or exposure to environmental toxicants. Oxidative stress is one of the main causes of DNA damage, and p53 involvement is therefore certain. With both pathways activated by oxidative stress, the resulting regulated crosstalk between p53 and Nrf2 will either encourage protective production of antioxidants by the Nrf2 pathway or p53-induced apoptosis. The following discussion focuses on the interactions between both pathways leading to the desired outcome.

4.1 Nrf2 stress response

The transcription factor Nrf2 has a short half-life under normal conditions, as it is ubiquitinated in the Cul3–Keap1 ubiquitin E3 ligase complex and subsequently degraded into proteasomes. Keap1 (Kelch ECH associating protein 1) is a well-studied protein containing a total of 27 cysteine residues (human). Three residues, C151, C273 and C288 have been shown to react with various oxidants and electrophiles *in vitro*. The modification of the residues can lead to conformational changes in the KEAP1 protein and a decrease in Cul3-Keap1 ubiquitin E3 ligase activity. It may therefore play a crucial role in Nrf2 stabilization, although the exact mechanism is not yet known. However, modification of Keap1 is not the only means of inhibiting Nrf2 degradation. Studies have also shown that several proteins, p21 and p62 among them, can block interactions between Keap1 and Nrf2 (explained later in this section). After stabilization, Nrf2 forms a complex with sMAf proteins that together bind to the domain of the antioxidant response element (ARE), resulting in the expression of several antioxidant target genes (Kansanen *et al.,* 2013).

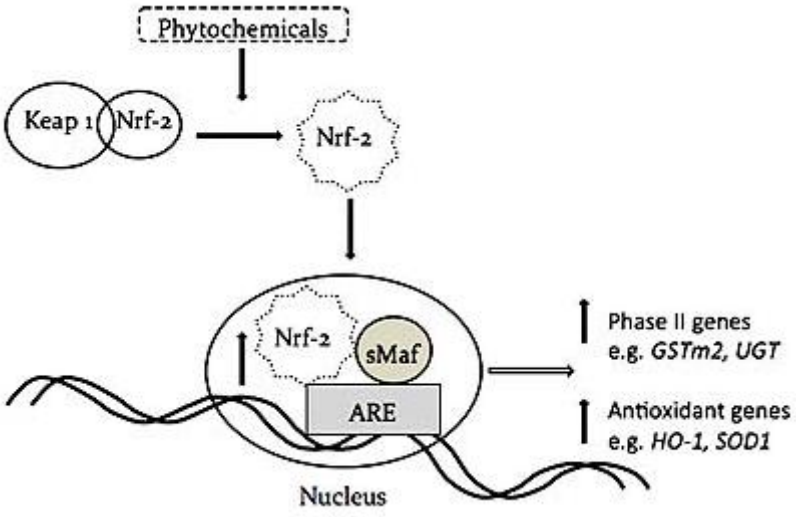

Figure 7: *Nrf2 activation after exposure to oxidative stress (Saw et al., 2011)*

4.2 Influence of Nrf2 on the p53 pathway

To date, there is hardly any information available about any possible regulation of p53 signaling by the Nrf2 pathway. You *et al.* (2011) provides some evidence that Nrf2 could contribute to the expression of MDM2 through the antioxidant response element and act therefore as a negative regulator of p53. In a study on Nrf2-deleted murine embryonic fibroblasts (MEFs), they found a significant decrease in MDM2 expression. This would make sense if the production of anti-ROS enzymes by the Nrf2 pathway is disturbed by the apoptosis-inducing ROS enzymes of the p53 pathway (Wakabayashi *et al.,* 2010).

4.3 Influence of p53 on the Nrf2 pathway

As the target genes of p53 and Nrf2 can have opposite functions, it is not surprising that several studies detected an inhibitory function of p53 on the target genes of Nrf2. Faraonio *et al.* (2006) noted inhibition of three Nrf2 target genes that have an important role in the antioxidant response: x-ct, Nqo1 and Gst1a1. Their main function is to neutralize ROS, therefore opposing the apoptosis inducing function (through ROS) of p53 (Faraonio *et al.,* 2006). The mechanisms underlying the p53-dependent inhibition of Nrf2 target genes is still to be discovered (Wakabayashi *et al.,* 2010).

4.4 Indirect interaction through p21

P21 is one of the main target genes of the p53 response pathway inducing cell-cycle arrest, but it also plays a role in other responses, such as cell differentiation, senescence, apoptosis and DNA repair. Moreover, it has been shown to have cell protective functions when exposed to oxidative stress (Wakabayashi *et al.,* 2010). Chen *et al.* (2012) found a novel interaction between p21 and Nrf2 that might explain these protective functions. Their study showed that *in vitro* p21 could modify Nrf2 directly and thus disturb its ubiquitination and proteasomal degradation by Keap1. Moreover, *in vivo* models showed a higher concentration of Nrf2 protein in p21 wild type mice (p21 +/+) than in p21 mutant mice (p21-/-), further supporting the hypothesis that p21 is a positive stimulator of Nrf2 (Chen *et al.,* 2012) (Wakabayashi *et al.,* 2010).

4.5 Balance between p53 and Nrf2

As with the HIF1 pathway, the interactions between Nrf2 and p53 can lead to different responses. Again, cell fate is probably determined by the severity of the stressor, in this case oxidative stress. Therefore, the following model can be proposed (figure 6) (Wakabayashi *et al.,* 2010). Substantial oxidative stress can lead to severe DNA-damage, thus inducing strong p53 response. P53 suppresses antioxidant gene expression in the Nrf2 pathway, further increasing ROS concentrations and thereby producing a strong apoptotic signal through the up-regulation of pro-apoptotic factors.

When, however, oxidative stress is only mild, p53 induction will be weaker, generating a milder stress response. P53 response genes such as p21 are activated, leading to cell-cycle arrest. The interruption of the cell-cycle allows p21 to stabilize Nrf2 by inhibiting its interaction with Keap1. The resulting Nrf2 response leads to transcription of the antioxidant genes protecting the cell against the oxidative stress. Moreover, Nrf2 causes even further down-regulation of p53 by enhancing MDM2 expression, thus ensuring cell survival.

Unfortunately, a gray area in this model relates to cases when induction of p53 and Nrf2 is moderate. The model proposes that in this case, both pathways will find a middle ground and the cell will go into senescence. The induction of p21 will lead to cell-cycle arrest and further activation of the Nrf2 pathway to prevent apoptosis through ROS. Since however, the cell is also exposed to a moderate amount of stress, p53 will target response genes switching the cell into terminal differentiation, hence avoiding further proliferation.

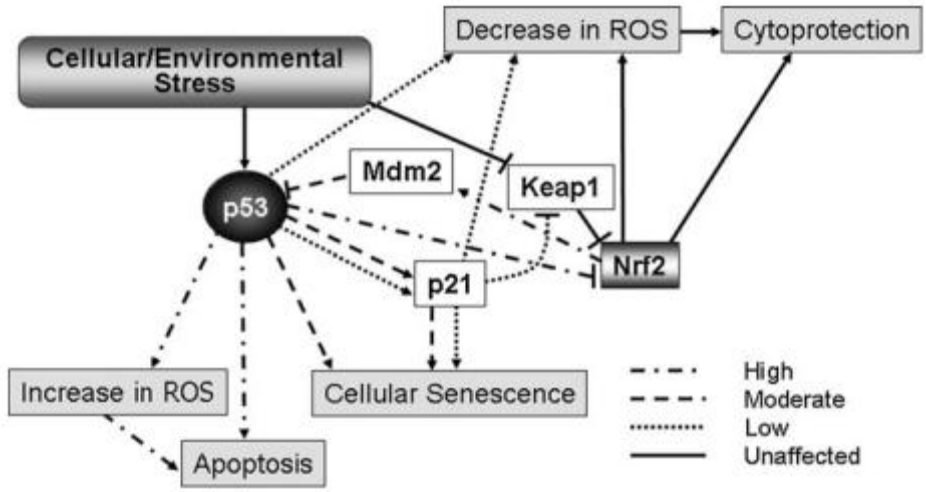

Figure 8: *Proposed interaction between p53 and Nrf2 under conditions of oxidative stress (Wakabayashi et al., 2010).*

5. Determining cell fate during ER stress

The endoplasmatic reticulum (ER) is the primary location for protein synthesis and modification. ER malfunctioning can result in the misfolding of proteins, potentially giving rise to such serious diseases as cystic fibrosis and hypercholesterolemia. ER exposure to stress may therefore be very dangerous (Cullinan & Diehl, 2006). The ER stress pathway, known as the unfolded protein response (UPR), is a protective pathway that can alternate the expression of important genes involved in protein modification as well as induce apoptosis. Although direct involvement of p53 has not been found, the apoptotic function of the ER stress pathway suggest a possible interaction.

5.1 ATF4 stress response

Exposing the ER to stress causes UPR sensors to trigger signaling molecules activating transcription factor 6 (ATF6), inositol requiring 1 (Ire1) and PKR-like endoplasmic reticulum kinase (PERK). Both ATF6 and Ire1 modulate gene expression by changing the transcriptional program. PERK, however, reduces protein translation to protect the cell from any further damage by inducing phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) (Cullinan & Diehl, 2006). Activating transcription factor 4 (ATF4) is one of the few target genes of eIF2α triggered during ER stress (Yang *et al.,* 2016). It is a transcription factor causing up-regulation of several genes involved in amino acid transport and synthesis. Importantly, it induces transcription factor C/EBP homologous protein (CHOP), which is a well-known pro-apoptotic protein (Szegezdi *et al.,* 2006). It is also thought to co-act with p53, as it can up-regulate pro-apoptotic genes and inhibit anti-apoptotic genes in the intrinsic apoptotic pathway (Engel *et al.,* 2013).

5.2 Influence of ATF4 on the p53 pathway

Although a direct connection between ATF4 and p53 has still to be discovered, Engel *et al.* (2013) proposes an indirect influence of ATF4 through CHOP that seems to contradict its apoptotic function. The study found that, since up-regulation of CHOP correlated with up-regulation of MDM2 and its coinciding inhibition of p53 activation, it possibly has a pro-survival function (Engel *et al.,* 2013). In contrast, Mihailidou *et al.* (2010) proposed a pro-apoptotic connection between CHOP and p53, noting that CHOP inhibited p21 and its anti-apoptotic function. By doing so, the pro-apoptotic response of p53 was enhanced and cell death was promoted (Mihailidou *et al.,* 2010).

6. Discussion

To conclude, the literature provides plenty of information on interactions between the transcription-factor mediated pathways p53, HIF1 and Nrf2. A few suggestions of interplay between p53 and ATF4 were also found. The results show that the switch between pro-survival and pro-death signaling depends on the nature of the p53 response, which directly correlates with the nature and duration of the stressor, as a result of the promoter selectivity of p53 created by posttranslational modifications (Kruse & Gu, 2009). The p53-triggered switch between cell survival and apoptosis is essentially a factor of the crosstalk with other stress response pathways. P53 can either suppress or stimulate the other pathways, depending on the p53 response itself. Since, for example, p21 is a positive stimulator of the Nrf2 pathway (Chen *et al.,* 2012), p53-induced protective responses involving p21 (e.g. the induction of cell-cycle arrest) could possibly stimulate other stress response pathways, such as Nrf2. This would make sense, as reversible cell-cycle arrest gives other pathways time to repair damage caused by a stressor. For similar reasons, all p53 protective stress responses might be expected to stimulate other pathways, while destructive responses should inhibit them. Although such inhibitory functions of p53 have been found with regard to all three pathways, the exact mechanisms remain unclear. Further research needs to reveal if and how stimulation of the destructive p53 response actually inhibits the other pathways.

The interaction between p53 and the other pathways appears to be reciprocal. Notably, HIF-1, Nrf2 and ATF4 can all influence p53 by targeting its main inhibitor MDM2. However, the nature of their influences differ. Nrf2 and ATF4 (through its target gene CHOP) increase MDM2 expression and therefore decrease p53 activation (Faraonio *et al.,* 2006) (Engel *et al.,* 2013). HIF-1, on the other hand, can bind MDM2 and therefore inhibit its function of de-activating p53 (Chen *et al,* 2003). Another study found that, since HIF-1 and p53 require the same co-activators, they could oppose each other in a competition for transactivation of their target genes (Schmid *et al.,* 2004). The result of this competition would depend on the available concentration of the transcription factor, which correlates with the pathway activity. The cytoplasmic concentration of the transcription factor would then be the decisive factor in determining the direction of crosstalk between p53 and the other pathways. The balance between the strength of the p53 response and the other responses is essential for determining which pathway dominates the regulation. In a model proposed by Sermeus & Michiels (2011) for hypoxia, the strength of the p53 response directly correlates with the nature and duration of the stressor. If such a model can also be applied to the other stress response pathways, it would theoretically imply that weak or mild stressors lead to domination of HIF-1, Nrf2 and ATF4 over p53. In these cases, cellular response will be pro-survival. When the stressor is very strong or its duration excessively long, p53 will prevail over the other pathways. Whether the cell will go into a pro-survival or pro-apoptotic state will then depend on the p53 response itself.

Interestingly, a similar trend in the expression of genes in the HIF-1, Nrf2 and ATF4 pathway was observed in our own research on the viability of PCKS during 48h incubation. Although not all of the results were statistically significant, the significant results revealed an initial up-regulation that decreased over time. Notably, the concentration of the p53-regulated pro-apoptotic gene BAX exhibited an increase over time during incubation. This might suggest that, when initially exposed to stress, the cell-protective pathways HIF-1, Nrf2 and ATF4 try to adapt the cell to the stressor. However, correspondingly with the model mentioned above, duration of stressor exposure can change the balance among pathways. Over time, p53 becomes more influential (as can be seen in the

resulting up-regulation of BAX) and start to pre-dominate over the other pathways, which would explain the gradual decrease in up-regulation of these other pathways.

If the diminished viability of PCKS can be explained by an increased dominance of p53 over time, this process could possibly be manipulated to extend slice lifespan. Although the interactions are not completely understood, MDM2 could be an important target gene in such a manipulation, as HIF-1, Nrf2 and ATF4 all can mediate their influence on p53 through this ubiquitin ligase.

Although this potential sample maintenance strategy remains mere speculation, it can be said with certainty that no simple interaction involved in the interplay between p53 and the other pathways, but a dynamic crosstalk involving multiple exchanges. The results of previous research indicate that this crosstalk furthermore changes over time, along with the corresponding influence on the PCKS viability. Moreover, the review of this research provided by this study demonstrates how the interactions between p53 and the other pathways have only been examined separately. Such a procedure assumes that the p53 response is the decisive factor in determining cell fate. Although this hypothesis is very likely, mutual interactions between the other stress response pathways cannot be excluded.

Understanding the interactions between p53 and the HIF-1, Nrf2 and ATF4 pathways constitutes an important step towards prolonging the viability of PCKS. However, more information is required in order to fully connect theory with reality. As the duration and nature of the stressor is essential for pro-survival/pro-apoptotic responses within the cell, the stress to which the PCKS are exposed during incubation should be identified and characterized. This can be done by using specific biomarkers that are only expressed during certain types of stress. For example, Oude Avenhuis (2016) found that PCKS are possibly damaged by oxidative stress due to a decrease in GSH/GSSG ratio. Influencing the interaction by increasing MDM2 levels will prevent p53 inhibition of Nrf2 as well as increase activation of p21, stimulating the protective response of the cell. If hypoxia and ER stress can be identified during PCKS incubation, their interaction with p53 can be manipulated in a similar fashion. Whether such manipulations are desirable, remains questionable. After all, the cell might be damaged beyond repair and an enhanced protective response cannot prevent the cell from proliferating. PCKS viability might therefore still decrease due to the spread of malfunctioning cells. Fighting the oxidative stress response in PCKS by influencing the p53-Nrf2 ratio is an important first step towards finding the answer to this question, as the viability of the manipulated PCKS and the normal PCKS could be compared.

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