Perspective

The role of p53 in reproduction, an unexpected function for a tumor suppressor

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At the beginning of the 21st century, the role of p53 as a tumor suppressor was well established; however, the function of p53 other than tumor suppression, especially its function under physiological conditions, was poorly understood. Here, we revisited the journey of a multidisciplinary team of researchers that revealed an unexpected and important function of p53 in regulating maternal reproduction at that time. This finding of p53 in maternal reproduction helped us gain important insights about the p53 protein and expanded our understanding of p53 protein functions in addition to its tumor suppressive function. This finding also provided a possible clue to explain the observed evolutionary selection on some alleles of the p53 pathway.

Since the discovery of p53 in 1979 as a 53-kD cellular protein that binds to SV40 large T antigen, tremendous studies have been done to understand the role of p53 in cancer. At the beginning of the 21st century, it was clear that p53 is a tumor suppressor protein. Loss of p53 function is often a prerequisite for tumor initiation and progression. p53 is the most frequently mutated gene in human cancer. Around 50% of human cancers harbor p53 mutations (Freed-Pastor and Prives, 2012; Muller and Vousden, 2014). In human cancers containing wild-type p53, the p53 signaling is often dysfunctional (Levine et al., 2006; Liu et al., 2014). Mice deficient for p53 are prone to developing tumors, which lead to a reduced lifespan (Donehower et al., 1992). In humans, Li–Fraumeni syndrome patients with germline p53 mutations have an increased cancer risk and develop different types of cancer at an early age (Bougeard et al., 2008).

It was also clear that p53 is a stress sensor. In normal cells and tissues under non-stressed conditions, p53 protein is maintained at a low level due to a very short protein half-life. In response to a wide variety of stress signals, including DNA damage, oncogene activation, nutrient stress, and ROS, p53 is stabilized and its protein levels quickly increase in cells (Levine et al., 2006; Vousden and Prives, 2009). p53 functions as a sequence-specific transcription factor, which induces a list of its target genes, including p21, Puma, Fas, etc. These protein products of p53 target genes are involved in many important cellular functions, including apoptosis, cell cycle arrest, senescence, and DNA repair, which contribute to the tumor suppressive function of p53 (Levine et al., 2006; Vousden and Prives, 2009; Feng and Levine, 2010). Therefore, p53 can ensure the replication fidelity and maintain genomic stability to prevent tumor development.

It was at this time we joined Dr Arnold Levine (Arnie)'s group for postdoctoral training. Since many of p53's functions in tumor suppression had been revealed through the identification of its target genes, we set out to search for new p53 target genes with the goal to further understand the mechanism of p53 in tumor suppression. The consensus p53 DNA-binding sequence had been identified and characterized as a tandem of two decameric palindromic sequences RRRC(A/T)(T/A)GYYY$(N)_{0\text{-}14}$ RRRC(A/T)(T/A)GYYY, in which R stands for purine, Y stands for pyrimidine, and N stands for any nucleotide (el-Deiry et al., 1992). With the completion of the genome sequence, it was possible to use computational methods to search for potential p53 target genes. p53MH, a computer algorithm, was developed by Dr Ott's group in collaboration with Arnie, which can search for potential p53 target genes by identifying putative p53 DNA-binding sites on a genome-wide scale (Hoh et al., 2002).

With the aid of the p53MH algorithm, we started the search for novel p53-regulated genes. One of the genes we identified was leukemia inhibitory factor (LIF). We validated that p53 protein physically binds to the LIF promoter region containing the putative p53 binding site predicted by the p53MH by employing chromatin immunoprecipitation assays. The p53 binding site in the LIF promoter region confers p53-dependent transcriptional activity as examined by luciferase reporters assays. Further, p53 can regulate both basal and inducible transcription of LIF. These results validated LIF as a previously unidentified p53 target gene (Hu et al., 2007).

We then tried to figure out what function of p53 was mediated by its transcriptional regulation of LIF. LIF is a pleiotropic cytokine that has broad biological functions; LIF can induce the differentiation of murine M1 myeloid leukemia, maintain mouse embryonic stem cell pluripotency, etc. (Yue et al., 2015). LIF also plays an important role in embryonic implantation. LIF expression levels in uterine transiently increase at the time of implantation to regulate several events during implantation, including stromal decidualization and the synthesis of prostaglandins, an important mediator for implantation and decidualization (Horita et al., 2007; Salleh and Giribabu, 2014). While mice deficient for LIF are viable, LIF deficiency causes infertility of female mice due to defects in embryonic implantation (Stewart et al., 1992).

It had been known that p53-null mice are challenging to breed. Arnie's lab maintained p53-null mouse colonies with two different genetic backgrounds, in which female p53-null mice appeared to be very inefficient in getting pregnant. The Jackson Laboratory also suggested breeding p53-null mice using heterozygous instead of homozygous females, while males can be either heterozygous or homozygous. Angie Teresky, who maintained mouse colonies for Arnie's lab, kept detailed breeding records of p53-null mice. The analysis of breeding of p53-null mice from her records clearly showed that in two inbred strains (C57BL/6J and 129SV^{sl}), p53-null female but not male mice showed impaired fertility with a decreased pregnancy rate and reduced litter size (Hu et al., 2007). These results indicated that p53 is required to maintain maternal reproduction, and loss of p53 impairs maternal reproduction. Since LIF deficiency was known to cause defects in embryonic implantation and infertility of female mice, we suspected that p53 is involved in the regulation of LIF in uterine during implantation to ensure the proper implantation and fertility. We examined LIF expression levels in the uterine of female mice with different p53 status and found that p53-null female mice have lower LIF levels in uterine, especially at the time of implantation when transiently induced high levels of LIF are crucial for embryonic implantation (Hu et al., 2007). We further found that indeed p53-null female mice displayed impaired implantation (Hu et al., 2007). Importantly, administering recombinant LIF protein to the pregnant p53-null female mice at the implantation stage restored maternal reproduction by improving implantation (Hu et al., 2007). Our experiments further showed that p53 and estrogen receptor α are activated in endometrial tissues during implantation to coordinately regulate LIF production (Feng et al., 2011). Taken together, these results revealed an unexpected yet important function of p53 that is not directly related to its tumor suppressive function.

It is worth noting that the regulation of transient activation of p53 in endometrial tissues during implantation is still not well understood. Interestingly, we found that LIF negatively regulates p53 protein levels and functions in cancer cells (Yu et al., 2014). LIF can activate the Stat3 signaling pathway, which in turn induces the expression of ID1, the helix–loop–helix protein inhibitor of differentiation and DNA binding. ID1 increases MDM2 expression to accelerate p53 protein degradation (Yu et al., 2014). It is therefore possible that p53 and LIF form a negative feedback loop, which is important for the transient activation of p53 and transient induction of LIF during implantation.

While p53 had been studied extensively for its role in tumor suppression and the finding of p53 in maternal reproduction appeared to be a surprise for those of us who viewed p53 as a tumor suppressor, there were indications that p53 could have functions in normal cellular processes in addition to tumor suppression. At the time, Arnie established the Simons Center for Systems Biology at the Institute for Advanced Study, Princeton to host a diverse group of theo retical physicists, cancer biologists, mathematicians, and computational biologists. This group of scientists embraced the enormous amounts of biological/genetic data generated by recent technologies and clinical data to conduct research at the interface of molecular biology and the physical sciences. They developed algorithms to study evolutionary genetics, molecular profiling of cancer, patterns of reassortment in viruses, neoantigen fitness in prediction of response to checkpoint blockade immunotherapy, etc. Dr Gurinder (Mickey) Atwal, a member of the Simons Center for Systems Biology at the time (currently an Associate Professor at Cold Spring Harbor Laboratory), analyzed the haplotype structure of a cluster of single nucleotide polymorphisms (SNPs) of MDM2, a key negative regulator of the p53 protein, in human populations with different ethnic backgrounds. In humans, naturally occurring SNPs with functional consequences exist in genes at critical nodes in the p53 pathway. E3 ubiquitin ligase MDM2 is the most important negative regulator of p53 (Hu et al., 2012). In the MDM2 gene, a common SNP SNP309 with a T-to-G change in the regulatory region in the first intron of the gene creates a stronger binding site for transcription factor Sp1, which results in the increased transcription levels of MDM2 and the attenuation of p53 function (Bond et al., 2004). Humans with the G allele of SNP309 have an increased risk for cancer development (Bond et al., 2004). Mickey found that some alleles of MDM2 containing SNP309 are under evolutionary selection (Atwal et al., 2007). A similar observation was made with p53 codon 72 SNP, a functional SNP with a G-to-C change that results in either an arginine (R72) or a proline (P72) at codon 72 of the p53 protein. The P72 allele has a weaker p53 activity and function in tumor suppression (Jeong et al., 2009). It has been indicated that p53 codon 72 SNP may modify cancer risk, but consensus has not been reached yet on this in the literature (Whibley et al., 2009). It has been reported that the allele frequency of the p53 codon 72 SNP differs among human populations with different ethnic backgrounds, as well as different latitude and winter temperature where people live, which suggests that certain alleles containing p53 codon 72 SNP are under evolutionary selection (Basu and Murphy, 2016). These observations

suggest that the human p53 pathway is under evolutionary selection.

The tumor suppression function of p53 is unlikely a cause for the evolutionary selection giving that cancer is predominantly a disease that occurs late in lifespan and is much more common in older people. It is plausible that the p53 pathway is under evolutionary selection at least in part due to its regulation of embryonic implantation and reproduction. We therefore were interested in whether p53 regulates implantation and reproduction in humans. LIF is also important for implantation in humans (Aghajanova, 2004). We found that p53 codon 72 SNP modulates the transcriptional regulation of LIF by p53; the P72 allele shows a weaker transcriptional activity towards LIF (Kang et al., 2009). This result indicates that different alleles of SNPs that modulate the p53 pathway could have an impact on implantation and reproduction in humans. To address this question, Arnie led us to collaborate with Dr Zev Rosenwaks, a Professor of Obstetrics and Gynecology and Reproductive Medicine at Weill Cornell Medical College, who is renowned for his pioneering work in assisted reproductive technology. This was an exciting collaboration between a systems biology group and physicians specialized in reproduction studying the role of p53 in human reproduction. We analyzed the SNP genotypes of a list of SNPs in the p53 pathway in *in vitro* fertilization (IVF) patients and found that the p53 P72 allele is enriched in IVF patients and is a risk factor for implantation failure (Kang et al., 2009). We observed additional selected alleles of SNPs in the p53 pathway, including SNPs in LIF and MDM2 genes that are enriched in IVF patients (Kang et al., 2009). These results suggest that p53 is involved in the regulation of human reproduction, which could be an important cause for the evolutionary positive selection of some alleles in the p53 pathway.

Interestingly, the involvement in reproduction has been observed with other two p53 family members, p63 and p73. p63 is an ancestral member of the p53 family during evolution. p63 has two groups of isoforms (TAp63 containing the N-terminal transactivation TA domain and ΔNp63 lacking the TA domain). TAp63 is functionally and structurally similar to the p53 protein. In mice, TAp63 is expressed in the nuclei of oocytes and plays an important role in maintaining the genome integrity of female germ cells during meiotic arrest (Suh et al., 2006). TAp63 senses DNA damage in oocytes and induces p53-independent apoptosis to efficiently eliminate damaged oocytes, which in turn protects the genome stability of female germ cells (Suh et al., 2006). p73 also regulates maternal reproduction. TAp73 plays an important role in maintaining the fidelity of female germ cells and proper ovary functions (Tomasini et al., 2008). Loss of TAp73 in female mice leads to a decreased primordial and primary follicular pool size, reduced ovulatory ability, and poor quality of oocytes with increased aneuploidy and decreased developmental competence (Tomasini et al., 2008). Female TAp73-deficient mice are infertile (Tomasini et al., 2008). These findings made from mouse models demonstrate the role of p53 family proteins in reproduction.

Therefore, we further studied the involvement of p63 and p73 in human reproduction through collaboration with the Systems Biology group and Dr Rosenwaks' group. Dr Asad Naqvi and Dr Haijiang Wang, members of the Simons Center for Systems Biology, analyzed the haplotype structure of a cluster of SNPs of human p63 and p73 genes and found that some SNPs in p63 and p73 genes appear to be under evolutionary selection (Feng et al., 2011). Interestingly, there is a clear enrichment of selected alleles of these SNPs in IVF patients, especially those with advanced maternal age (Feng et al., 2011). It is worth noting that IVF patients with advanced maternal age are more likely to have decreased fertility due to the poor oocyte quality and impaired ovary function. These observations support the involvement of p63 and p73 in human production through maintaining oocyte quality and ovary function.

The p53 family is evolutionarily conserved. The homologues of the p53 family genes exist in many different organisms, including simple organisms that do not develop cancer within their short lifespan (Belyi et al., 2010). Although most of studies on p53 center around its tumor suppressive function, tumor suppression may not be the primordial function of the p53 family proteins. Our journey studying the role of p53 in reproduction helped us gain important insights about the p53 protein and expanded our understanding of the p53 protein in addition to its tumor suppressive function. These studies also provided clues to explain the observed evolutionary selection on some alleles of the p53 pathway. This set of studies could not have been done without the collaboration of a multidisciplinary team of researchers, including cancer and molecular biologists, systems biologists, and reproductive medicine physicians. We were fortunate to be members of this team. We experienced the power of integrating large amounts of data/information from sequencing using systems biology approaches to probe biological systems. We learned that while p53 was extensively studied for its role in cancer, tumor suppression does not have to be the only or primordial function of p53. That is exactly what many recent studies have shown that p53 is involved in cancer as well as other diseases and many physiological functions, such as metabolic diseases, aging, and neurodegenerative diseases (Vousden and Lane, 2007; Chang et al., 2012; Kung and Murphy, 2016). It was truly a fun and exciting journey, which led to the finding of an unexpected yet important p53 function.

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