

# Regulation of Fertility by the p53 Family Members

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## Abstract

The p53 family members, which consist of 3 transcription factors—p53, p63, and p73—are conserved during evolution. The p53 family proteins are involved in many important cellular functions, including tumor suppression (p53 and p73), the development of epithelial cell layers (p63), and the development of central nervous system and immune system (p73). Studies on p53-like proteins in low organisms have demonstrated that their primordial functions are to maintain the genomic integrity of germ cells and ensure faithful development and reproduction. In vertebrates, the p53 family proteins retain these functions in reproduction and at the same time have developed additional important functions in reproduction, such as the regulation of embryonic implantation (p53). p53 regulates embryonic implantation through transcriptional regulation of leukemia inhibitory factor (LIF). p63, in particular TAp63, is a main regulator to protect the fidelity of female germ cells during meiotic arrest. p73, in particular TAp73, regulates the ovary function and the quality of oocytes. Loss of p53, p63, or p73 genes in female mice leads to a significant decrease in fertility. These functions of the p53 family proteins in reproduction provide a plausible explanation for positive evolutionary selection observed in a group of single nucleotide polymorphisms and haplotypes in the p53 family genes. A better understanding of the functions of the p53 family proteins in reproduction may lead to new strategies for fertility treatment.

**Keywords:** p53, p63, p73, implantation, female germ cells

## Introduction

The p53 family proteins consist of 3 transcription factors: p53, p63, and p73. These 3 proteins have a very similar domain organization. They all contain an amino-terminal transactivation domain (TAD), a proline-enriched region (PR), a central DNA binding domain (DBD), and a carboxy-terminal oligomerization domain (OD). p63 and p73 proteins have additional domains, including a carboxy-terminal sterile- $\alpha$  motif (SAM), which is thought to mediate protein-protein interactions, and a transcription inhibition domain (TID).<sup>1</sup> The p53 family proteins have limited overall homology but share strong similarity in the DBD (approximately 55%–58% between p53 and p63/p73 and approximately 87% between p63 and p73).<sup>2</sup> The p53 family proteins bind to similar or identical DNA sequences to regulate the transcription of a subset of genes in common and a subset of genes unique to each protein, which in turn are involved in similar as well as unique cellular functions. p53, p63, and p73 are all involved in

tumor suppression. In addition, p63 is essential for epidermal morphogenesis and limb development, and p73 is involved in the development of central nervous system and immune system. Studies on p53-like proteins in low organisms, including *Caenorhabditis elegans* and *Drosophila*, have revealed that the primordial functions of p53 family proteins are to maintain germ cell integrity and faithful reproduction. Recent studies in mice and humans have demonstrated that the p53 family proteins play important roles in regulation of reproduction, including maintaining primordial and primary follicular pool size, germ cell genomic integrity, ovulatory ability, pre-implantation development, and embryonic implantation. The regulation of reproduction appears to be an evolutionarily conserved and important function of the p53 family proteins.

## The Isoforms and Functions of the p53 Family Proteins

The p53 family proteins exist as multiple functionally diverse proteins that

originate from a combination of alternative promoter usage and alternative mRNA splicing.<sup>3–7</sup> Each gene has 2 promoters: the first is located in a noncoding region upstream of exon 1, and the alternative promoter is located in intron 4 of the p53 gene and intron 3 of the p63 and p73 genes, respectively. The promoter upstream of exon 1 generates the transactivating (TA) isoforms, which are transcriptionally proficient, and the alternative promoter leads to the expression of amino-terminal truncated ( $\Delta$ N) isoforms that lack the transactivation domain and are transcriptionally inactive. For both p53 and p73, additional N-terminal variants are generated by alternative splicing or internal initiation of translation.<sup>8</sup>  $\Delta$ N isoforms can actively repress the transactivation of

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TA isoforms by competing for binding to the DNA binding elements in regulated genes, and thus they function as dominant negative inhibitors of TAp53, TAp63, and TAp73. To add further complexity, splice variants of the C-terminal region generate 3 additional isoforms of p53 and p63 ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) and at least 8 splice variants of p73 ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\phi$ ). Splicing at the C-terminal end of the p53, p63, and p73 genes gives rise to isoforms that regulate stability of the protein and DNA binding. Thus, p53, p63, and p73 each have many different isoforms with a number of diverse properties. The full-length and truncated isoforms of the p53 family generally exhibit reciprocal biological functions:  $\Delta N$  isoforms support proliferation, whereas TA isoforms promote cell cycle arrest, cellular senescence, and apoptosis.<sup>8</sup> The existence of p53 family proteins with opposing activities implies that the balance of various isoforms may determine cell fate.

p53 protein plays an important role in tumor suppression. In response to a variety of stress signals, p53 is activated and transcriptionally induces its target genes that lead to various cellular responses, including cell cycle arrest, apoptosis, and senescence, to inhibit the proliferation of nascent cancer cells.<sup>9</sup> The p53 gene is the most frequently mutated gene in human cancer; ~50% of human cancers have mutations in the p53 gene, and ~80% of human cancers have dysfunction of the p53 pathway.<sup>9</sup> p63 and p73 are also involved in tumor suppression. Both TAp63 and TAp73 isoforms can bind to canonical p53 DNA binding elements and transcriptionally activate a subset of p53-target genes, which induce p53-like functions including cell cycle arrest, apoptosis, and senescence.<sup>3,6,10,11</sup> A study examining the tumorigenic effects of p63 and p73 in heterozygous mouse models showed that p63<sup>+/-</sup> and p73<sup>+/-</sup> mice are predisposed to develop spontaneous tumors. p63 and p73 also cooperate with p53 in tumor suppression. p53<sup>+/-</sup> p63<sup>+/-</sup> and p53<sup>+/-</sup> p73<sup>+/-</sup> mice have higher tumor burden and

increased metastasis compared with p53<sup>+/-</sup> mice.<sup>12</sup> Loss of TAp73 *in vivo* leads to increased incidence of spontaneous and carcinogen-induced tumors, suggesting that TAp73 is a tumor suppressor. In human tumors, although mutations of p63 and p73 are rare, overexpression of  $\Delta N$  isoforms of p63 and p73 has been often observed.<sup>13-15</sup> It has been suggested that this imbalance between TA and  $\Delta N$  isoforms could inhibit the function of TA isoforms in tumor suppression, which promotes tumorigenesis.

Although the p53 family proteins are all involved in the regulation of tumor suppression, the most important role of p63 and p73 appears to be the regulation of differentiation and development. p63 has been shown to be essential for epidermal morphogenesis and limb development. Mice deficient for all p63 isoforms die within hours of birth, presumably due to dehydration. The developmental abnormalities of these mice include craniofacial malformations, limb truncation and lack of epidermis, and squamous epithelia (prostate, urothelium) and epithelial appendages, such as hair follicles, teeth, and mammary, salivary, and lachrymal glands.<sup>16,17</sup> In humans, germ line mutations in the p63 gene cause several rare autosomal dominant developmental diseases, including ectrodactyly ectodermal dysplasia-clefting syndrome (EEC), acro-dermato-ungual-lacrimal-tooth malformations (ADULT), limb-mammary syndrome (LMS), Hay-Wells syndrome (AEC), split-hand/foot malformations (SHFM), and Rapp-Hodgkin syndromes.<sup>18-20</sup> These diseases are characterized by various degrees of limb abnormalities, ectodermal dysplasia, and facial clefts, indicating the relevance of p63 to normal epidermal development in humans.<sup>21</sup> p73 is involved in the regulation of neuronal development and differentiation and of immune function. Mice deficient for all p73 isoforms are viable but are runt and have high rates of mortality. A majority of the p73 null mice die before 4 weeks of age, and only 25% of them survive to adulthood. These mice

exhibit profound developmental defects, including hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation, and abnormalities in pheromone sensory pathways.<sup>22</sup> It has been shown that  $\Delta N p73$  is expressed in developing brain, sympathetic ganglia, and adult neurons and plays an important role in neuronal survival during development and in adult neurons by blocking p53/TAp63/TAp73-mediated apoptosis.<sup>22-24</sup> In addition, TAp73 is important for the maintenance of neural stem cell (NSC) pool and may support neuronal differentiation.<sup>25</sup> Increased expression of TAp73 has been observed in NSCs following differentiation and in neuroblastoma cells that have been induced to differentiate by retinoic acid.<sup>26</sup> Ectopic TAp73 overexpression can induce neuronal differentiation, probably by antagonizing Notch signaling.<sup>27</sup>

### The Evolution of the p53 Family Genes

The p53 family is evolutionarily conserved. The homologues of the p53 family genes have been described in many different organisms, including molluscs, sea anemone, clams, *C. elegans*, *Drosophila*, frogs, and zebrafish.<sup>28-35</sup> The DBD is the most conserved domain among different organisms. All p53-like family proteins that have been identified so far contain a DBD. The residues in DBD that contact DNA and the DNA binding sequences are highly conserved. All members of the p53-like family proteins that have been investigated so far demonstrate very similar DNA binding specificities.<sup>36,37</sup> Based on sequence similarity, p53-like family proteins in primitive organisms and invertebrates, including sea anemone, *C. elegans*, and *Drosophila*, are more closely related to p63/p73 than to p53.<sup>38,39</sup> Furthermore, many p53-like family proteins identified in invertebrates contain a SAM domain, which is a protein interaction domain existing in p63/p73 protein but not in p53 protein. Therefore, p63/p73 is likely to be evolutionarily ancient, and p53 is possibly evolved from a p63/p73-like

gene.<sup>29</sup> The high conservation of residues that contact DNA and of the DNA binding specificity suggests that the original function of this protein family reaches far back in evolution.

The existence of ancient p53 homologue proteins in organisms that have no threat from cancer within their short life spans suggests that the primordial functions of the p53 family proteins may not be tumor suppression, and the tumor suppression function may be derived from their primordial functions. Evidence suggests that maintaining the genomic integrity of germ cells to ensure the faithful reproduction could be an important primordial function of the p53 family proteins. In sea anemone (*Nematostella vectensis*), the p53-like family protein nvp63 is highly expressed in germ cells but not in somatic cells. nvp63 is activated by genotoxic stress signals to induce apoptosis in germ cells, which in turn eliminates damaged germ cells. In this way, nvp63 maintains the integrity of the genome transmitted to the next generation and avoids defective progeny.<sup>30</sup> *C. elegans* contains a single p53-like family protein, CEP-1 (*C. elegans* p53-like-1). CEP-1 is commonly expressed in germ cells. In response to genotoxic stress, CEP-1 induces 2 target genes that encode BH-3 (Bcl-2 homology 3) domain-only proteins, EGL-1 (egg laying abnormal-1) and CED-13 (cell death abnormality protein 13), to activate apoptosis in germ cells and eliminate defective offspring from the population.<sup>32,40-42</sup> In response to UV, CEP-1 can induce phg-1 (pharynx associated Gas1), which is a cell cycle inhibitor that results in the arrest of germ cell proliferation to allow time for DNA repair. In addition, CEP-1 is required for normal chromosome segregation during meiosis in germ cells.<sup>32</sup> In *Drosophila*, the only p53-like family protein, Dmp53 (*Drosophila* p53), also plays a similar role in female germ cells. Dmp53 is highly expressed in germ cells.<sup>40</sup> In response to DNA damage and other genotoxic stress, Dmp53 protein is activated and induces apoptosis in primordial germ cells. Dmp53 null mutants

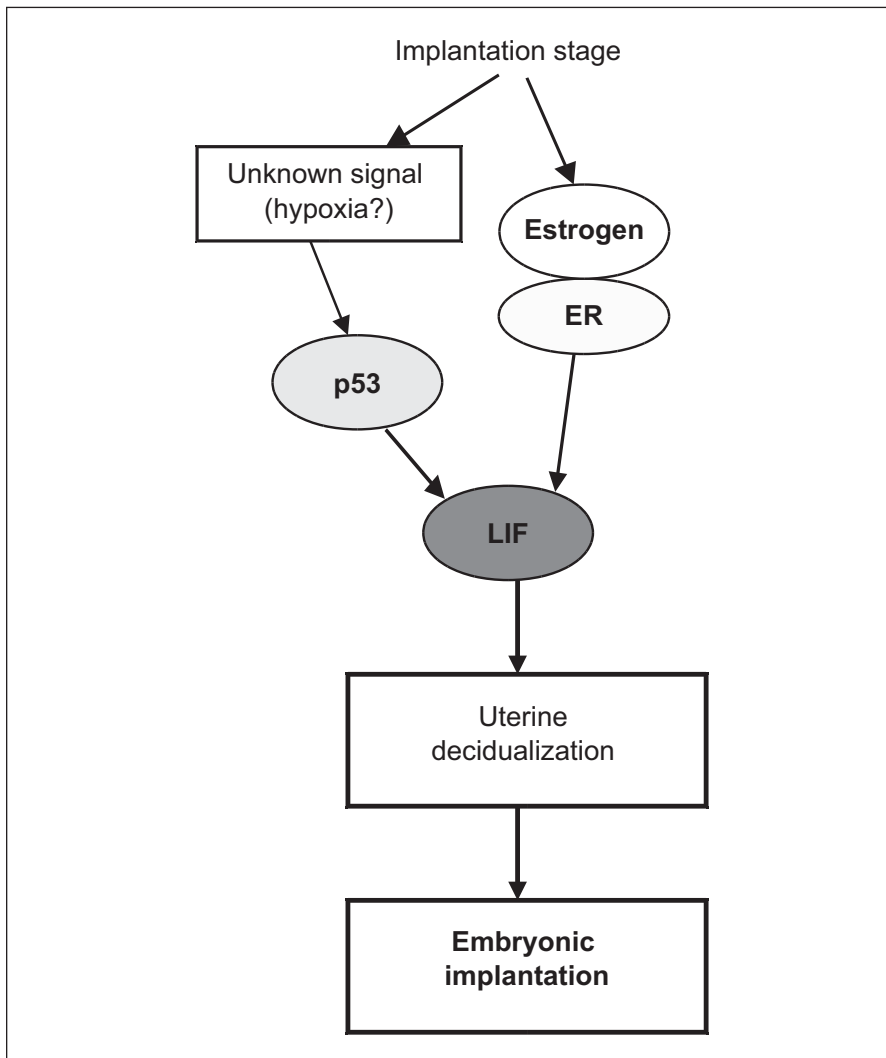
exhibit genomic instability, especially after  $\gamma$ -irradiation.<sup>43</sup> In *Drosophila*, caspases that cause apoptosis are normally maintained in an inactive state by inhibitors of apoptosis (IAPs). In response to apoptotic stimuli, IAP antagonists, including reaper, hid, grim, sickle, and jafra2, are transcriptionally activated to degrade IAPs and induce apoptosis. Among them, reaper, sickle, and hid are Dmp53-regulated genes.<sup>36,43,44</sup> Another important pathway in Dmp53-induced apoptosis is the Hippo (Hpo) pathway, which includes the kinases Hpo and Warts/Lats. It has been shown that in response to irradiation, Hpo is activated by phosphorylation, which in turn promotes apoptosis, in a Dmp53-dependent manner.<sup>45</sup> In addition to apoptosis, Dmp53 may be involved in DNA repair. It has been suggested that a group of DNA double-strand break (DSB) repair genes, including Ku70, Ku80, Mre11, and Rad50, are potential target genes of Dmp53.<sup>44,46</sup> Dmp53 is also required for proper meiotic recombination. Dmp53 null mutants have reduced meiotic recombination frequencies.<sup>47</sup> It has been shown that Dmp53 proteins are activated by the DSBs in oocyte precursors during the initiating steps of meiotic recombination. In cells that are defective for meiotic DNA repair, Dmp53 is persistently activated. Furthermore, Dmp53 is involved in the development of the ovary. Dmp53 null mutants have degenerative ovarioles and decreased fertility compared with their parental strains.<sup>48</sup> It is clear from these observations that the maintenance of genomic integrity of germ cells to ensure faithful development and fecundity is an important primordial function of the p53 family proteins.

### The Role of the p53 Protein in Reproduction in Mice

The primordial function of the p53 family proteins is evolutionarily conserved. Similar function of the p53 family proteins in reproduction is observed in mice. In mice, p53 protein is highly expressed in testes with increased

activity during spermatogenesis, and it regulates both spermatogenesis and radiation response. Meiotic recombination appears to be an important signal to activate p53 protein during spermatogenesis. p53 is transiently activated in early spermatocytes; the expression of p53 peaks between the leptotene and zygotene stages and disappears after the early pachytene stage.<sup>47</sup> p53 null mice or mice with reduced levels of p53 have a high frequency of multinucleated giant cells within the testicular seminiferous tubules, which is a degenerative syndrome most likely due to the impaired meiotic divisions during the meiotic prophase.<sup>49</sup> p53 also mediates DNA damage-induced spermatogonial apoptosis.<sup>50</sup> Irradiation can induce p53 activation in spermatocytes, which results in apoptosis to remove damaged spermatocytes.<sup>51,52</sup> In p53 null mice, irradiation induces very limited apoptosis, which in turn results in the production of an increased number of giant-size spermatogonial stem cells. p53 protein is also expressed in mouse embryos during development to monitor and respond to DNA damage. p53 is highly expressed in mouse embryos until the midgestation stage.<sup>53</sup> In response to irradiation, p53 protein is activated to induce apoptosis to efficiently eliminate the damaged offspring, whereas p53 null embryos show a very small percentage of death, which results in a high percentage of developmental abnormalities.<sup>54,55</sup> These observations demonstrate that the p53-dependent DNA damage response (apoptosis) is highly active in germ cells and during development to ensure faithful development and fecundity in mice.

Mammals have a much longer and more complex gestation period compared with other vertebrates. The p53 family proteins gained new functions in regulation of reproduction in mammals during evolution. Our recent work has revealed an additional important function of p53 in reproduction in mice; p53 regulates embryonic implantation through its target gene, leukemia inhibitory factor (LIF) (Figure 1).<sup>56,57</sup> Although



**Figure 1.** p53 regulates embryonic implantation through LIF in mice. p53 is selectively activated in endometrial tissues at the implantation stage. Estrogen levels also increase at the implantation stage, which in turn activates the estrogen signaling pathway in endometrial tissues through ER $\alpha$ . Selective activation of p53 and ER $\alpha$  coordinately regulates LIF expression at the implantation stage to induce uterine decidualization and ensure embryonic implantation.

p53 null mice seem to be fertile and are able to give birth of intact offspring, detailed analysis of fertility has shown that p53 loss in female but not in male mice significantly decreases fertility. p53 null female mice have much lower pregnancy rates and smaller litter size. This function of p53 in regulation of maternal reproduction is mediated by LIF. LIF is a multifunctional cytokine that plays a crucial role in embryonic implantation. LIF functions through its

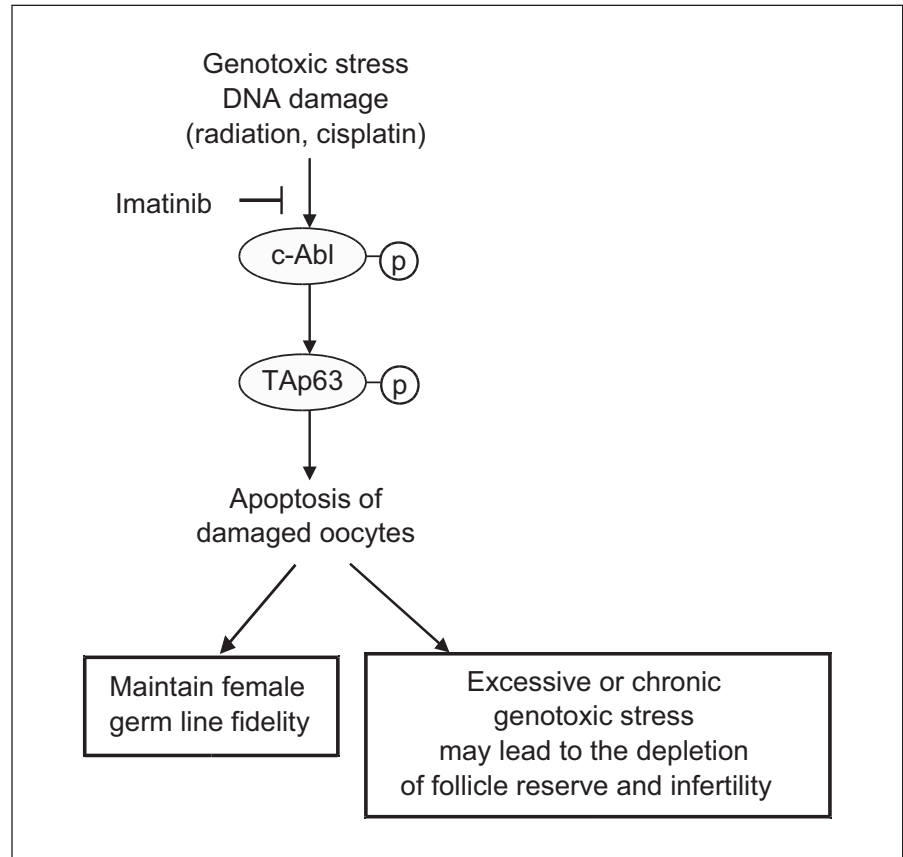
binding to the LIF receptor complex, composed of LIF receptor (LIF-R) and glycoprotein gp-130, which in turn activates selective pathways, including JAK/STAT3, MAPK, and PI3K/ATP pathways.<sup>58,59</sup> At the onset stage of implantation, the LIF expression levels increase significantly in the endometrial glands to induce decidualization of uterine tissue.<sup>60-62</sup> Decidualization is a process that changes endometrium into decidua by modifying

endometrial stromal cells, uterine glands, and vessels as well as population of uterine immune cells, thus preparing the uterus for embryonic implantation. LIF-deficient female mice have a defect in reproduction due to the complete lack of uterine decidualization at the implantation stage that leads to the failure of blastocyst implantation. This reproduction defect in LIF-deficient female mice can be rescued by injection of exogenous LIF at the implantation stage.<sup>61</sup> The increased uterine LIF expression at the implantation stage requires the coordinated regulation of p53 and estrogen.<sup>63</sup> p53 can bind to the p53-binding element in the first intron of mouse LIF gene and regulate the expression levels of LIF under both non-stressed and stressed conditions. At the implantation stage, p53 protein levels and activity are increased selectively in the endometrial tissues, including endometrial glands, where the highest levels of LIF expression are observed. It is unclear what signals activate p53 in endometrial tissues at the implantation stage. Implantation occurs under the hypoxic condition,<sup>34</sup> and hypoxic signals are known to activate p53.<sup>35</sup> It is possible that a hypoxic condition is the stimulus for p53 activation in the uterus at the implantation stage. Another regulator of LIF expression is estrogen. The estrogen levels and the activity of estrogen receptor, which mediates the estrogen signaling, increase at the implantation stage in parallel with the increase of LIF expression.<sup>64</sup> In primary cultured mouse endometrial epithelial cells, the highest expression levels of LIF were observed in p53 wild-type cells in the presence of estrogen, suggesting the coordinated regulation of LIF by p53 and by estrogen signaling. In p53 null mice, uterine LIF levels at the implantation stage are much lower than those in wild-type mice, which results in impaired implantation. The implantation can be restored in p53 null female mice by injection of exogenous LIF at the implantation stage, which greatly improves fertility in these mice.

## The Role of p63 Protein in Protecting Female Germ Line Fidelity in Mice

As described above, p63 is a more ancestral member of the p53 family during evolution. Phylogenetic analysis consistently show that the single p53-like family gene in genetically tractable organisms such as *C. elegans* or *Drosophila* is related to the p63 gene.<sup>38</sup> In mice, the central role of p63 in epidermal development, which is contributed mainly by  $\Delta N$  isoforms of p63, was established by the observation made in mice null for both TA and  $\Delta N$  isoforms. Using mice that were deficient only for TAp63 isoforms, studies have shown that TAp63 is responsible for maintaining genomic integrity of oocytes through eliminating damaged oocytes.<sup>65</sup> This function of TAp63 in mammalian female germ cells is similar to the function of the p53-like family proteins in *C. elegans* and *Drosophila*, which is consistent with the notion that p63 is the ancestral member of the p53 family.

In mice, TAp63 $\alpha$  plays an important role in maintaining the genome integrity of female germ cells during meiotic arrest. TAp63 is mainly expressed in the nuclei of oocytes but not in male germ cells. The expression of TAp63 $\alpha$  in oocytes is initiated in the late prophase I from late pachytene stage at embryonic day 18.5 and peaks in the diplotene stage shortly after birth.<sup>65,66</sup> TAp63 $\alpha$  is activated in oocytes in response to DNA damage induced by  $\gamma$ -irradiation and chemotherapeutic agents, such as cisplatin. TAp63 $\alpha$  is activated through phosphorylation by c-Abl, a nonreceptor tyrosine kinase, which in turn induces a group of proapoptotic genes, including Puma and Noxa, and leads to p53-independent apoptosis to efficiently remove damaged cells (Figure 2).<sup>67</sup> Loss of p63 in oocytes prevents the cleavage of caspases-9 and caspases-3. The oocytes without TAp63 or p63 are completely resistant to DNA damage-induced apoptosis.<sup>65,68</sup> These results clearly demonstrate that TAp63 retains the primordial



**Figure 2.** TAp63 protects female germ cells in response to genotoxic stress. In response to genotoxic stress signals, TAp63 is activated through phosphorylation by c-Abl. Activated TAp63 induces p53-independent apoptosis to remove damaged cells and protect the fidelity of female germ cells. Under the conditions of excessive or chronic genotoxic stress, TAp63-dependent apoptosis may lead to the depletion of follicle reserve and infertility. The activation of TAp63 and TAp63-dependent apoptosis can be blocked by imatinib, the c-Abl kinase inhibitor.

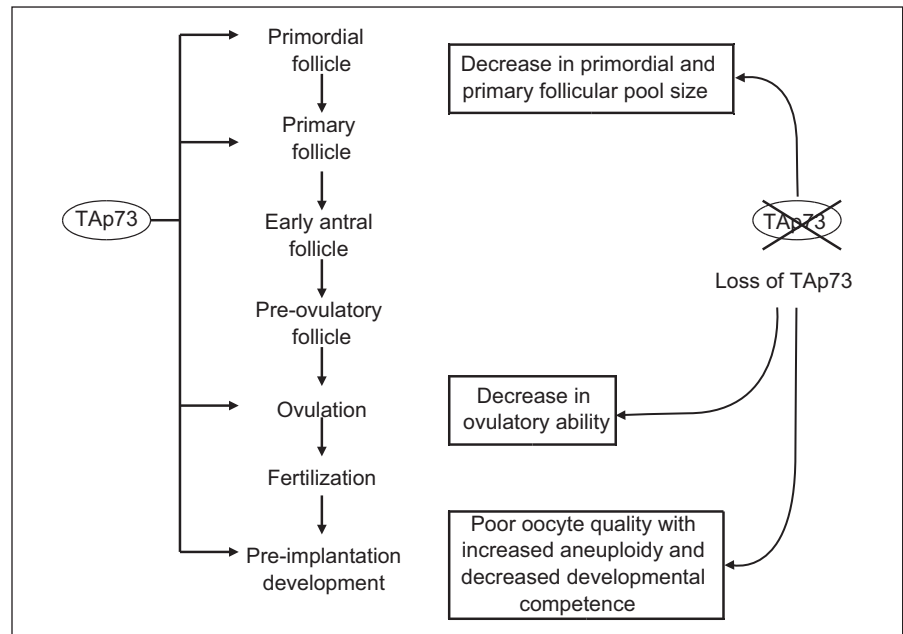
function of the p53 family proteins in mice, which protects the female germ line fidelity by eliminating damaged cells and, therefore, controls maternal reproduction.

## The Role of p73 in Ovary Function and Quality of Female Germ Line Cells

Studies on mice deficient for p73 have shown that p73 regulates reproduction at several different steps.<sup>22,69</sup> Mice deficient for both TA and  $\Delta N$  isoforms of p73 are infertile. As described above, p73 is involved in the regulation of neuronal development and differentiation. Among many neuronal defects, p73 null mice

have a dysfunction of the vomeronasal organ, an accessory olfactory structure involved in pheromone detection, which normally expresses high levels of p73.<sup>22</sup> Mice deficient for p73 are not interested in mating with sexually mature females and are infertile. Mice null for TAp73 only are infertile in female mice but through different mechanisms. TAp73-deficient mice have defects in several steps in maternal reproduction, including reduced follicular pool size, decreased ovulatory ability, and poor oocyte quality (Figure 3).<sup>69</sup> First, the number of primordial (quiescent) and primary (early growing) follicles is significantly decreased in TAp73-deficient ovaries. Because the duration of female fertility is determined

by the initial size of the primordial follicular pool and by the rate of its activation and subsequent depletion, the lower number of primordial follicles in TAp73-deficient mice results in a faster reproductive failure. Second, TAp73-deficient female mice have a reduced ovulation rate. These female mice ovulate much fewer oocytes compared with wild-type mice. Furthermore, these few ovulated oocytes are trapped under the bursa and cannot progress toward the fallopian tubes as they should. Third, TAp73-deficient oocytes are of poorer quality than wild-type oocytes. It has been reported that when germinal vesicle oocytes as well as ovulated oocytes from 3- to 4-week-old mice were induced to mature *in vitro*, TAp73-deficient oocytes exhibited a striking increase in aneuploidy and spindle abnormalities, including multipolar spindles, spindle relaxation, and spindle scattering accompanied by varying degrees of misalignment. During meiosis and mitosis, accurate chromosome segregation is critical to prevent aneuploidy. The spindle assembly checkpoint (SAC) complex plays an important role in ensuring accurate chromosome segregation by sensing the improper attachment of sister chromatids to the mitotic or meiotic spindle and delaying anaphase until all chromosomes are correctly oriented for segregation. SAC complex contains over 20 protein partners, including Bub1, Bub3, BubR1, MAD2, cyclin B, Rae1, and Aurora B. Defect of proteins in the SAC complex, such as Bub1, BubR1, or Aurora B, has been reported to cause an increase of aneuploidy, which contributes to poor oocyte quality and infertility.<sup>70-72</sup> It has been shown that TAp73 is able to directly interact with several partners of the SAC complex (Bub1, Bub3, and BubR1) and is involved in SAC complex localization and activities. In ovulated oocytes, TAp73 is localized in the cytoplasm and associated specifically with the meiotic spindle at metaphase II. In TAp73-deficient oocytes, the localization of Bub1 and BubR1 to the meiotic spindle and its associated chromosomes is diminished. BubR1, an important component of the



**Figure 3.** TAp73 regulates ovary function and quality of female germ cells. TAp73 is involved in several steps to regulate female reproduction, including maintaining the sizes of primordial and primary follicular pool, the proper ovulatory function, and genomic integrity of female germ cells. Loss of TAp73 results in reduced follicular pool size, decreased ovulatory ability, and poor oocyte quality.

SAC complex, is a protein kinase that binds and inhibits p55cdc20 (p55), a major anaphase-promoting complex regulatory protein, to control the activation of the anaphase-promoting complex.<sup>73</sup> TAp73 is found to be able to potentiate BubR1 activity; loss of TAp73 compromises BubR1 function. The weakened SAC response in TAp73-deficient oocytes could be an important mechanism for aneuploidy and genomic instability that accounts for infertility in females. The poor quality of TAp73-deficient oocytes has been demonstrated also by their poor developmental competence. Comparing the ability of TAp73-deficient and wild-type oocytes to undergo *in vitro* fertilization (IVF) and preimplantation development showed that TAp73-deficient oocytes often fail in preimplantation development. Although the fertilization rate is similar in both genotypes, embryos obtained from TAp73-deficient oocytes often arrest during early cleavage, resulting in embryos with multinucleated blastomeres and blastocysts of inferior quality with abnormal cell number.<sup>69</sup> Together, TAp73 regulates female reproduction

through maintaining the fidelity of female germ cells and proper ovary functions (Figure 3).

Increased maternal age has a significant negative impact upon fertility, and a major underlying mechanism for decreased fertility in aged females is the poor quality of oocytes.<sup>74</sup> Interestingly, the expression of TAp73 in oocytes declines with natural aging. Furthermore, oocytes from aged wild-type mice and young TAp73 knockout mice exhibit similar abnormalities, including increased aneuploidy and spindle abnormalities,<sup>69</sup> suggesting that TAp73 is involved in maintaining genomic stability in oocytes, and decreased TAp73 function may contribute to the increased genomic instability and aneuploidy observed in aged normal oocytes that have compromised developmental capacity.

### The Role of p53 and Its Pathway in Implantation and Fertility in Humans

In humans, p53 appears to retain its function in embryonic implantation

and is involved in the regulation of reproduction. The regulation of LIF by p53 is conserved from mice to humans.<sup>56</sup> As in mice, in humans the LIF gene is a p53 target gene, which contains a p53 DNA-binding element in the first intron. p53 transcriptionally regulates LIF expression levels under both nonstressed and stressed conditions. LIF is also an important player in implantation in humans. The levels of LIF protein increase at the time of expected implantation in fertile women. It has been shown that women with higher endometrial LIF levels during midsecretory phase are more likely to get pregnant than are women with lower LIF levels.<sup>75</sup> Decreased uterine LIF levels are often associated with decreased fertility in humans; women with unexplained infertility have lower LIF levels compared with fertile women.<sup>76,77</sup> Furthermore, high endometrium LIF levels are associated with a successful implantation after an IVF cycle in IVF patients.<sup>78</sup> The regulation of human LIF gene by p53 suggests that p53 may regulate the efficiency of implantation and reproduction in humans.

In humans, naturally occurring single nucleotide polymorphisms (SNPs) with functional consequences exist in genes at critical nodes in the p53 pathway. p53 codon 72 SNP is a common coding SNP in the p53 gene that results in either an arginine (R72) or a proline (P72) residue at codon 72. The p53 P72 allele is weaker in inducing apoptosis and suppressing cellular transformation compared with the p53 R72 allele.<sup>79</sup> Individuals with the p53 P72 allele have increased cancer risk compared with the p53 R72 allele.<sup>79</sup> The allele frequency of this SNP varies among populations with different ethnic backgrounds and also changes as a function of the latitude where people live. The p53 P72 allele is an ancestral allele with ~60% frequency in African populations. However, in Caucasian and Asian populations, which arose more recently, the p53 P72 allele becomes the minor allele, with ~30% frequency. The change of the allele frequency of this SNP in the p53 gene suggests that certain alleles are under

**Table 1.** Significant Association of Selected Alleles of Genes in the p53 Family Pathways with Decreased Fertility in Humans

	Gene (SNP)	Genotype	Control, %	IVF patients			
				<35 years		≥35 years	
				%	<i>P</i> <sup>a</sup>	%	<i>P</i> <sup>a</sup>
The p53 pathway	p53 (rs1042522)	G	77.3	64.8	1.8E-06 <sup>b</sup>	70.3	0.03 <sup>b</sup>
		C	22.7	35.2		29.7	
	MDM2 (rs2279744)	T	62.7	55.7	0.03 <sup>b</sup>	58	0.35
		C	37.3	44.3		42	
	MDM4 (rs1563828)	C	68.4	61.7	0.05 <sup>b</sup>	59	0.02 <sup>b</sup>
		T	31.6	38.3		41	
HAUSP (rs1529916)	G	68.5	56	0.004 <sup>b</sup>	65.1	0.46	
	T	31.5	44		34.9		
LIF (rs929271)	T	71.4	63.3	0.008 <sup>b</sup>	68.9	0.43	
	G	28.6	36.7		31.1		
p63	p63 (rs17506395)	T	70.9	78.3	0.02 <sup>b</sup>	79.2	0.01 <sup>b</sup>
		G	29.1	21.7		20.8	
p73	p73 (rs4648551)	G	56.9	58.4	0.70	66.5	0.004 <sup>b</sup>
		A	43.1	41.6		33.5	
	p73 (rs6695978)	G	95.8	94.7	0.31	92.1	0.03 <sup>b</sup>
A		4.2	5.3		7.9		

Note: IVF patients were recruited at Weill Cornell Medical College. DNA from 200 healthy Caucasian individuals was obtained from the Coriell Cell Repositories, and women recruited as controls for the Women's Insights and Shared Experiences (WISE) study were used as controls.

<sup>a</sup> $\chi^2$  test.

<sup>b</sup>Significant difference between IVF patients and controls.

evolutionary selection pressure depending upon the ethnic background. Cancer arises late in life, so it is therefore unlikely that the tumor suppression function of p53 is the major cause for the observed evolutionary selection. Instead, the role of p53 in implantation and reproduction is more likely to be the cause for the observed evolutionary selection. The p53 P72 allele has lower transcriptional activity toward a subset of p53 target genes compared with the p53 R72 allele. We found that LIF is one of the p53 target genes that are differentially induced by the p53 codon 72 SNP. The p53 P72 allele has ~2-fold lower transcriptional activity toward the LIF gene compared with the p53 R72 allele in cultured human cells.<sup>80</sup> Recently, mice carrying SNPs at p53 codon 72 have been generated by knocking in wild-type human p53 gene carrying

either the p53 P72 allele or R72 allele in place of the corresponding mouse p53 gene.<sup>81</sup> Interestingly, the p53 P72 allele produces much less uterine LIF protein in mice, especially at the implantation stage, compared with the p53 R72 allele.<sup>63</sup> This impact of the p53 codon 72 SNP upon the uterine LIF production suggests that this SNP may affect implantation and fertility in humans. Indeed, the association of the p53 P72 allele with decreased reproduction efficiency has been observed in IVF patients. Coulam and her colleagues reported that the p53 P72 allele is enriched in women with recurrent implantation failure.<sup>82</sup> Consistently, we found that the p53 P72 allele is enriched in IVF patients (Table 1) and is a risk factor for implantation failure after an IVF procedure.<sup>80</sup> Interestingly, it has been suggested that environmental

stresses, such as cold winter temperature, select the p53 R72 allele, which could increase the levels of LIF to promote a more efficient embryo implantation under adverse conditions.<sup>83</sup>

In addition to p53 codon 72 SNP, the p53 pathway contains other functional SNPs in genes that regulate the levels and activity of p53. Recently, we identified a SNP (SNP309) in the MDM2 gene, which encodes a key negative regulator of p53. SNP309 (a T to G change) in the regulatory region in the first intron of MDM2 gene creates a stronger Sp1 binding site, which results in 2- to 4-fold increased transcriptional levels of MDM2 and, therefore, the attenuation of p53. In humans, SNP309 is associated with increased risk for cancer.<sup>84,85</sup> MDM2 SNP309 also appears to be under evolutionary selection pressure, with its allele frequency differing greatly among populations with different ethnic backgrounds. The T allele is the ancestral allele with more than 90% frequency in African populations. However, in Caucasian and Asian populations, the frequency of the G allele, which arose more recently, is increased to ~40%. Similar evolutionary selection of certain alleles has been observed in MDM4, HAUSP (herpesvirus-associated ubiquitin-specific protease), and LIF genes.<sup>86-88</sup> MDM4 is a structural homolog of MDM2, which is also an important negative regulator of p53 through binding to the amino terminus of p53 protein. HAUSP is a deubiquitinating enzyme that plays a crucial role in regulating the levels of p53, MDM2, and MDM4. HAUSP binds and stabilizes p53 by deubiquitination. Meanwhile, HAUSP can also deubiquitinate Mdm2 and Mdm4 and down-regulate p53 levels.<sup>89</sup> Interestingly, selected alleles of MDM2, MDM4, HAUSP, and LIF genes have been found to be strongly associated with decreased fertility in IVF patients (Table 1).

Advanced maternal age is usually associated with decreased ovarian reserve and quality of oocytes and has a significant negative impact upon fertility. Impaired implantation is a more prominent cause for infertility in young patients with unexplained infertility

compared with patients of advanced maternal age. Interestingly, the association of these at-risk alleles in the p53 pathway with decreased fertility mainly occurs in young patients, and the association disappears or is largely reduced in patients with advanced maternal age (Table 1).<sup>88</sup> These observations strongly suggest a role of the p53 pathway in regulation of human reproduction through LIF and indicate the presence of candidate functional SNPs in these alleles that influence p53 function in reproduction. These observations also suggest that the regulation of reproduction is an important cause for the observed positive selection on certain alleles in the p53 pathway.

### The Roles of p63 and p73 in Reproduction in Humans

Although TAp63 has an important function in maintaining the fidelity of female germ cells in mice, TAp63 also plays a similar role in humans. Infertility in patients with p63 mutation has been reported. Four females from the same family with RHS syndrome due to a germline p63 mutation all exhibited premature menopause and infertility.<sup>90</sup> These patients have a heterozygous single nucleotide deletion at codon 595, within exon 14 of the p63 gene, which results in a modification of the reading frame and leads to a stop codon 65 bp downstream to the canonic TGA stop codon. These patients all have premature menopause at age of around 30.

The involvement of p63 and p73 in human reproduction has been further demonstrated by our recent study showing that the selected alleles of the p63 and p73 genes are associated with decreased fertility in IVF patients.<sup>63</sup> In human p63 and p73 genes, there are SNPs that appear to be under evolutionary selection pressure. These SNPs include a SNP in the p63 gene (rs17506395) and 2 SNPs in the p73 gene (rs4648551 and rs6695978). A clear enrichment of selected alleles of these SNPs has been observed in IVF patients (Table 1). Interestingly, the

at-risk alleles in the p63 and p73 genes for human fertility are enriched in patients of advanced maternal age, who are more likely to have decreased oocyte quality and ovary function. These data are consistent with the idea that p63 and p73 may play important roles in maintaining the quality of oocytes and ovary function in humans.

### Conclusion and Future Perspectives

The p53 family proteins are evolutionarily conserved, and an ancestral gene of p53/p63/p73 existed at early times during evolution. The primordial functions of this gene family are the surveillance of the genomic integrity of germ cells to ensure faithful development and reproduction. These functions appear to be evolutionarily conserved and can be found in mice and humans. In vertebrates, the p53 family proteins have developed additional important functions in reproduction, such as the regulation of embryonic implantation by p53. These functions of p53 family proteins in reproduction result in strong evolutionary selection pressure on this family of genes. Later in evolution, when the average lifetime of the individual exceeded the average lifetime of a cell, thus making tissue renewal mandatory, the new functions of the p53 family proteins were evolved, such as the tumor suppressive function of p53 and p73.

In humans, causes of infertility can be related to both male and female conditions. Approximately 60% of infertility is caused by female conditions. Among the causes of female infertility, ovulatory dysfunction is the single most frequent cause. The quality and quantity of the primordial follicle pool are a key of female fertility conservation. Oocytes exist as a limited population largely arrested in a potentially vulnerable, tetraploid state for a prolonged period. As women age, their ovary function declines and aneuploidy rate in oocytes increases. The effects of reproductive aging on women have become more important as women



increasingly become pregnant at older ages. Studies in mice have shown that TAp73 is involved in maintaining genomic stability in oocytes. TAp73 levels decline with aging, which may contribute to the increased aneuploidy in aged oocytes. It is currently unclear what causes the decrease of TAp73 expression with natural aging. It will be interesting to study whether increasing TAp73 levels in aged oocytes will improve ovary function and the quality of oocytes.

Preserving female fertility is also an increasingly urgent issue for female cancer patients. Ovarian failure and infertility are major adverse effects of anticancer therapy in children and young women. Chemotherapeutic agents can activate TAp63 in oocytes and induce apoptosis to remove damaged cells. This function of TAp63 in protecting the female germ line fidelity is crucial to ensure the integrity of the genome transmitted to the next generation; however, under conditions of excessive or chronic genotoxic stress, the same function of TAp63 may be responsible for the depletion of the oocyte population, which has a negative consequence of fertility. Germ cells are very sensitive to DNA damage insults. It has been shown that oocytes from mouse primordial follicles can be almost completely removed even at low doses of radiation.<sup>91</sup> A recent study demonstrated that the c-Abl kinase inhibitor imatinib, which blocks activation of TAp63, can prevent cisplatin-induced depletion of the follicle reserve and premature ovarian failure in mice (Figure 2).<sup>67</sup> It is possible that imatinib or other c-Abl inhibitors could be used to preserve female fertility during anticancer therapy. The challenging question is how to ensure that such treatment will not affect the antitumor activity of chemotherapeutic agents and at the same time not save damaged germ cells that may potentially increase mutation frequency. Further investigation is needed to gain a better understanding of the molecular mechanisms used by damaged germ cells to repair lesions and survive, which will help to develop effective approaches that preserve female fertility and at the

same time maintain the genomic integrity of germ cells during anticancer therapy.

Inefficient implantation is another important cause of infertility, especially in a portion of patients with unexplained infertility. Inefficient implantation is often a major cause of pregnancy failure after an IVF procedure. Administering recombinant human LIF may improve implantation in patients with inefficient implantation. Indeed, in a small group of patients with pure female factor infertility with unexplained recurrent implantation failure, the use of recombinant LIF in an IVF procedure improved implantation and increased pregnancy rates.<sup>92</sup> However, in an extended study, LIF treatment failed to show improved implantation in patients with implantation failure.<sup>93</sup> These inconsistent results suggest that multiple gene products and signaling pathways could be involved in the regulation of implantation. Therefore, it is important to carefully select patient groups who are more likely to have LIF deficiency for further study. Our finding that the p53 pathway regulates uterine LIF levels suggests that analyzing SNPs of the p53 pathway in IVF patients may help to identify patients who could potentially benefit from therapy with recombinant human LIF.

In summary, the discoveries of the functions of the p53 family proteins in maintaining female germ cell genomic integrity and regulating embryonic implantation have demonstrated that the p53 family proteins are important regulators of female reproduction. Further understanding of the functions of the p53 family proteins in reproduction could lead to the development of new strategies for fertility preservation and fertility treatment and could reduce the incidence of infertility.

#### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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