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Extra View

p53

A new player in reproduction

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Abbreviations: LIF, leukemia inhibitory factor; SNP, single nucleotide polymorphism; IVF, in vitro fertilization

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The roles of the p53 protein in tumor suppression have been firmly established. However, the functions of this protein under normal conditions or in the absence of stress, if any, have remained a mystery. In humans, some alleles containing a functional single nucleotide polymorphism in the p53 gene and its negative regulator, the Mdm2 gene, are under positive selection over evolutionary time frames, suggesting that the p53 pathway might have important functions that are optimized and selected for by evolutionary or reproductive pressures. Indeed, a recent study demonstrated a new function for the p53 protein in the regulation of maternal reproduction in mice, through transcriptional regulation of leukemia inhibitory factor (LIF), a novel p53 target gene. Sufficient uterine LIF levels are essential for the implantation of blastocysts or early embryos into the uterus. p53 deficient ($p53^{-/-}$) female mice have a reduced pregnancy rate and litter size, due to impaired implantation resulting from decreased uterine LIF levels. Administration of LIF to pregnant $p53^{-/-}$ mice restored maternal reproduction by improving implantation. An association has been reported between women carrying the p53 codon 72 polymorphism (a proline to arginine change) with recurrent implantation failure, suggesting a similar function for p53 in humans. These findings of a new function for the p53 protein in reproduction may help to explain the observed evolutionary selection of some alleles of the p53 and Mdm2 genes. This may also be an excellent example of antagonistic pleiotrophy.

The tumor suppressor p53 gene is known as "the guardian of the genome".[1,2] It plays a crucial role in maintaining genomic stability and tumor prevention. The p53 protein responds to a wide variety of stresses, such as DNA damage, telomere shortening, hypoxia, aberrant oncogene activation, or even nutrient depravation. Functioning as a sequence-specific transcription factor activated by these stresses, p53 initiates a transcriptional program, that can lead to apoptosis, cell cycle arrest or senescence in cells. This contributes to tumor suppression by either preventing or repairing genomic damage or eliminating potentially oncogenic clones of cells. In humans, over 50% of tumors contain mutations in the p53 gene and most of these mutations occur in the DNA-binding domain which eliminates transcriptional activity and is the most well conserved domain across different species.[3]

In humans, functional single nucleotide polymorphisms (SNPs) have been identified in both the p53 gene and its negative regulator, Mdm2, which can alter the levels or function of the p53 protein.[4,5] There is a common coding polymorphism in the p53 gene which results in either an arginine (Arg) or a proline (Pro) residue in the protein at codon 72. The proline 72 (P72) form of p53 protein is weaker than the arginine 72 (R72) form in inducing apoptosis and suppressing cellular transformation[6-8] while the p53 P72 polymorphism appears to be better at initiating senescense and cell cycle arrest.[9,10] The P72 allele is also associated in some tumors with an earlier age onset of tumor formation than the R72 allele and a less efficient response to chemotherapy in human populations.[11-13] In a study with elderly individuals (ages 70 to 80 years old) those individuals with an R/R72 genotype developed less cancer but died at a younger age than individuals with the P/P72 genotype.[14] This is yet another link between cancer and longevity. SNP309 has either a T or a G residue in the intronic promoter region of the first intron of the Mdm2 gene. The G-residue increases the binding affinity of the transcriptional activator, Sp1 and in turn results in an elevated Mdm2 expression and an attenuated p53 function.[5] Moreover, SNP309 is located in a region of Mdm2 promoter regulated by estrogen signaling, estrogen preferentially stimulates the transcription of Mdm2 from the SNP309 G allele and increases the levels of Mdm2 protein.[15] In humans, the SNP309 G allele is associated with an earlier age of onset of several cancers and an increased risk for tumorigenesis in a gender specific (females) and hormonal dependent manner.[5,16]

There are significant differences in allele frequencies for p53 codon 72 among individuals of different ethnic backgrounds. The P72 allele frequency is approximately 60% in African Americans and 30–35% in Caucasian Americans.[17] Furthermore, the P72 allele frequency is closely linked to latitude, increasing in populations as they near the equator.[18] This observation suggests that the codon

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72 polymorphism is evolutionarily balanced and maintained by natural selection that might be mediated by geographic factors such as temperature or sunlight or infectious diseases, etc. By comparison with chimpanzee DNA it is evident that the P72 allele is the older allele, currently present at a higher frequency in Africa while the R72 allele arose later in Caucasians and Asians. The frequency of Mdm2 SNP309 G allele also differs greatly among different ethnic backgrounds. The G allele frequency is approximately 43% in Caucasian Americans, and only 10% in African Americans. Even this 10% in African Americans appears to have been contributed by an admixture with Caucasians as demonstrated by its haplotype structure.[19] Thus it appears that, like the R72 polymorphism, the G-allele arose later in evolution with the migrations out of Africa of individuals who evolved into Caucasians and Asians and the T allele is ancestral to the G allele as supported again by comparison with chimpanzee DNA. This idea is reflected in the haplotype structures of the Mdm2 gene from African Americans, Caucasians and Asians. There are many different gene-wide haplotypes, and thus a large variability/diversity of haplotypes, associated with the T allele due to the presumption that the gene has undergone many recombination events during the long lifetime of the T allele in the population. On the other hand, there is very little diversity of G allele haplotypes found in Asian and Caucasian populations indicating that it arose recently in human evolution during which there was little time to accumulate recombination events.[19] However, assuming a neutral model, the severely reduced variability of G allele haplotypes would predict a much lower population frequency of the G allele than that currently observed in out-of-Africa populations. Considering the relatively high frequency of the common G allele haplotype in the Caucasian and Asian populations (43–48%) it might seem reasonable to suggest that a positive selection pressure has been exerted on the G allele in these populations, pushing the frequency towards fixation.

There exist a number of statistical tests for positive selection but most of these only utilize the frequencies of SNP variants and ignore haplotype structure and are better suited to detect selection over long evolutionary time scales (~10^{5-7} years for humans) where the selected allele has already been fixated in the population. The migration out of Africa is estimated to occur as recently as 40–50 thousand years ago, and thus it is necessary to adopt a different statistical approach to detecting such recent human evolution. To this end, we constructed a selection test premised on using an information-theoretic[20] measure of haplotype variability and thereby directly incorporating the information not only from the frequencies of SNPs but also the linkage disequilibrium between SNPs[19]. The resulting analysis of the haplotypes in Mdm2 provided significant quantifiable evidence for strong positive selection on the G allele of SNP309 in out-of-Africa populations. Developing such haplotype-based tests is an area of current active research since they have been shown to be much more powerful in detecting recent selective sweeps in the genome that have not yet reached fixation.[21]

This positive selection of the G allele might have come out as a consequence of the need to keep a balance between p53 activity and its negative regulator Mdm2 activity. As the p53 P72 allele changed to an R72 allele, p53 activity for some p53 regulated genes involved in apoptosis, prevention of tumors and responses to several DNA damaging drugs all improved because of increased transcriptional activity of the R72 allele compared to the P72 allele. At the same time the R72 allele decreased longevity in humans.[14] The Mdm2 gene T-allele to G-allele change acts as a balancing modifier of this change, increasing the concentration of Mdm2. Thus it might be that the T to G allele change in the Mdm2 gene is under positive selection in Caucasians and Asians so as to balance the increased p53 R72 activity that arose in those same populations. While this is a satisfying hypothesis it has a significant problem. Modern evolutionary theory suggests that the events in an individuals' life that occur in the post-reproductive years such as cancers and longevity are much less likely to have the kind of strong selective pressures observed with the haplotype structures (selection of the G-allele) of the Mdm2 gene. For this reason we considered two other possibilities for the functions of the p53 and Mdm2 genes in humans; (1) an involvement in reproductive processes and fecundity or (2) an involvement in responses to infectious diseases both of which have clearly shaped the human genome.

p53 is conserved from invertebrates to vertebrates, orthologs of p53 have been identified in *C. elegans*, Drosophila, zebrafish and frogs.[22-24] The existence of p53 in short-lived organisms with no occurring of cancers in the adult such as flies and worms, suggests that tumor suppression was not the original function for p53 and its pathway. There is some evidence for both the infectious disease and the fecundity hypotheses. One of the stress responses that activate the p53 pathway program is the production of nitric oxide which is commonly made during an inflammatory response.[25] This suggested the possibility that p53 is part of the innate immune system responding to inflammation and infections by initiating a program of defenses. Alternatively, the evolutionary origin of the p53 protein in lower organisms, utilizes its functions to protect the germ line from DNA damage and mutations. Indeed, in Drosophila and *C. elegans*, p53 is most commonly expressed in the germ cells and it functions in the surveillance of damaged germ cells to eliminate defective offspring from the population.[26,27] Therefore, the first functions of the p53 protein were to prevent developmental defects in the germ line. In Drosophila and *C. elegans* the only dividing cells in the adult organism are the germ line cells and a few "immune cells". The body plan for vertebrates, however, contains many tissues that continue to divide throughout a lifetime and renew themselves via tissue specific stem cells. Here, in higher organisms p53 has been recruited from the germ line to the stem cells and the dividing somatic cells so as to prevent cancers from arising in these organisms. It was this function of p53 as a tumor suppressor that was first detected in many somatic cells of the body.[28,29] This brings up an interesting question; does the p53 protein retain its ancient function in vertebrates for the surveillance of damage in the germ line? In *Xenopus laevis*, p53 dependent transcription is activated during early oogenesis and the levels of p53 remain relatively high during development. Inactivation of the p53 function prevents normal development in the Xenopus embryo.[30] In mice and rats, the p53 levels are very high during spermatogenesis. The p53 deficient mice or mice with reduced levels of p53 exhibit germ cell degeneration during the meiotic prophase, with a high frequency of multinucleated giant cells within the testicular seminiferous tubules.[31] p53 has also been suggested to mediate stress-related spermatogonial apoptosis after DNA damage.[32] In mouse embryos, both p53 mRNA and protein are expressed at a high levels until the midgestation stage,[33] and the p53-dependent DNA damage responses (transcriptional activation and apoptotic response) are

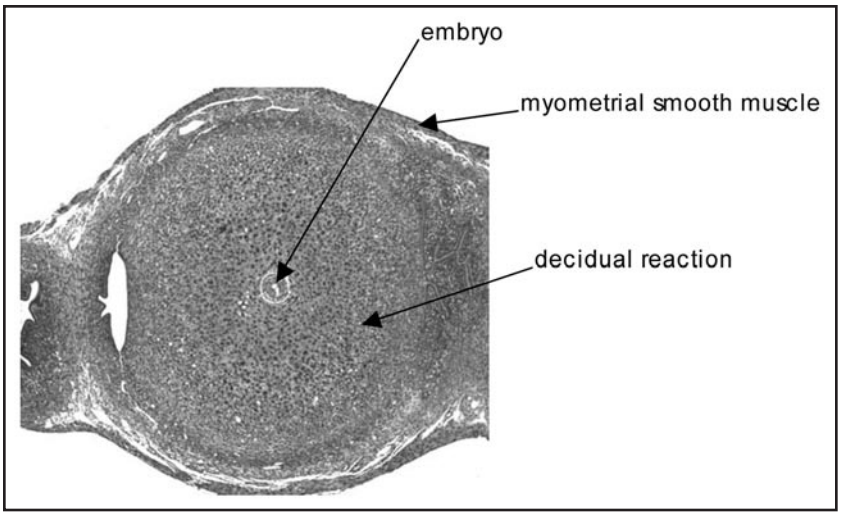

Figure 1. Wild type mouse uterus with an implanting embryo at day 7 of pregnancy. A uterus collected from wild type mouse with an implanting embryo at day 7 of pregnancy was fixed in 10% normal buffered formalin, embedded in paraffin, sectioned into 5-μM slices and stained with hematoxylin/eosin.

highly active throughout this period of development.[34,35] Wild type embryos treated with ionizing irradiation show a p53 dependent apoptosis, resulting in a high percentage of death to efficiently eliminate the damaged offspring, whereas $p53^{-/-}$ embryos have a very small percentage of death and a high percentage of developmental abnormalities.[35]

Recently, we found that the p53 protein has a normal physiological role in maternal reproduction through the regulation of implantation in mice. A significant decrease in the fertility rate was observed in p53 deficient ($p53^{-/-}$) female mice, but not male mice, with a poor pregnancy rate and a small litter size.[36] In C57BL/6J mice, while pregnancy rate and litter size are not affected when $p53^{-/-}$ male mice are mated with $p53^{+/+}$ female mice, the pregnancy rate and the litter size for $p53^{-/-}$ female mice in breeding pairs with $p53^{+/+}$, $p53^{+/-}$ or $p53^{-/-}$ males decrease dramatically and the decrease appears to be most severe when $p53^{-/-}$ females are mated with $p53^{-/-}$ males with the genotype of their embryos being $p53^{-/-}$. A similar observation was made with the $129SV^{sl}$ strain of mice, although the phenotype is less severe, suggesting there are strain-specific modifier genes that influence this function of the p53 protein.

The p53 protein mainly exerts its function through transcriptional regulation of its target genes. Therefore, an algorithm was employed that detects DNA sequences where the p53 protein is most likely to bind and activate transcription.[37] In this way a potential p53 target gene that was involved in maternal reproduction was identified, namely, leukemia inhibitory factor (LIF), a poly-functional glycoprotein cytokine, which plays an essential role in blastocyst implantation.[38] The p53 protein interacts with the p53 DNA binding elements identified by p53 algorithms in both human and mouse LIF genes in vivo as demonstrated by a chromatin immunoprecipitation (ChIP) assay. Luciferase reporter plasmids containing the putative p53 binding elements in the promoter region exhibited a p53-dependent transcriptional activity. p53 protein activation by several different stress signals significantly induced the expression of the LIF gene in both cell cultures and various mouse tissues, including spleen, thymus and uterus. Furthermore, endogenous p53 under no apparent stress conditions also had a significant impact on the basal transcription levels of LIF; with ~4 fold higher LIF expression levels in $p53^{+/+}$ cells compared with $p53^{-/-}$ cells.

LIF plays an important role in blastocyst implantation.[38,39] Implantation is a stage critical in mammalian embryonic development during which the blastocyst establishes a close interaction with the uterine tissues, which leads to the formation of the placenta to support the growth and development of the fetus. Figure 1 shows a wild type mouse uterus with an implanting embryo at day 7 of pregnancy. In many mammalian species, including mouse and human, LIF is most highly expressed at the onset of implantation.[40] In mice, this occurs at day 4 of pregnancy. Highly expressed in the endometrial glands, LIF protein is secreted into the uterine lumen and binds to its receptors on the surface of epithelial cells, preparing the uterus to be receptive to the implantation of the blastocyst. Sufficient levels of LIF are crucial for the initiation of implantation. $LIF^{-/-}$ female mice are infertile due to the defect in implantation. Injection of LIF to these mice at day 4 of pregnancy can initiate implantation and subsequent normal embryonic development to birth.[38,39] In $p53^{-/-}$ female mice, the expression levels of uterine LIF are significantly reduced, in the uterus from both non-pregnant mice and mice at day 4 of pregnancy. An impaired implantation function was also observed in $p53^{-/-}$ female mice. In these mice, the uterine development and estrous cycle hormonal levels appeared normal, the number of blastocysts produced and fertilized in the overducts were comparable to that in wild type mice and their morphology was normal. However, the number of implanted embryos was significantly reduced. Administering LIF to p53 deficient female mice at day 4 of pregnancy significantly increased the pregnancy rate and litter size with improved blastocyst implantation, which was not observed with wild type mice.[36]

LIF is an estrogen-responsive gene, and estrogen is involved in the regulation of the expression of uterine LIF, especially at day 4 of pregnancy.[39] Interestingly, while p53 is essential for maximal expression of LIF at day 4 of pregnancy and loss of p53 significantly reduced the expression of uterine LIF, no change in the levels and/or activity of p53 was observed during the pre-implantation period. Therefore, the expression of LIF was coordinated and regulated by both p53 and estrogen during implantation (Fig. 2). This regulation does not require the increase of the p53 levels that are observed in every other stress induced p53 response and may involve the interaction between p53, estrogen and estrogen receptor, which is a possible novel mechanism for p53 to regulate its target genes.

Besides a decreased fertility rate in $p53^{-/-}$ female mice, some embryonic defects are found in $p53^{-/-}$ mice, which are predominantly associated with females. This leads to a sex ratio distortion (more male mice born than females) in the p53 knockout mice. A substantial fraction of the female p53 null embryos exhibit a neural tube closure defect called exencephaly, with an outgrowth of neural tissue usually at the region of the fore- and mid brain.[41,42] Additional abnormalities in nulls including upper incisor fusion, ocular abnormalities and polydactyly of the hindlimbs have also been reported.[41]

The mechanisms for these fatal defects in a significant fraction of embryos in the absence of p53 are unclear but they are reminiscent of the function of p53 in fetal development in other organisms. While a LIF injection improved embryonic implantation in $p53^{-/-}$ female mice, similar birth defects were still observed in a substantial fraction (~30%) of mice born from $p53^{-/-}$ females with a LIF injection, indicating p53 plays a role in post-implantation development as well.[36]

While the importance of p53 in fecundity and development has been demonstrated, it is worth noting that the levels of p53 are highly important for developmental processes. Too much p53 protein also impacts development. This has been observed in $p53^{+/+}$ mice that are null for Mdm2, a key negative regulator of p53. Mdm2 inhibits p53 activity by regulating its location, stability and activity as a transcriptional activator. Mice lacking the Mdm2 gene are early embryonic lethal and this phenotype can be completely rescued by concomitant deletion of the p53 gene.[43,44] It is apparent that a fine balance of p53 levels appears to be critical for development, deviation from normal wild-type p53 levels in either direction can have serious detrimental consequences. Therefore, a possible explanation for the evolutionary positive selection observed with genes in the p53 pathway is keeping p53 levels and/or activity in an optimal range to maintain the normal development and reproductive fitness. This reinforces the p53 R72 and Mdm2 G-allele arguments presented previously. These studies also demonstrate the important role of p53 in fecundity a powerful phenotype for genetic selection of alleles.

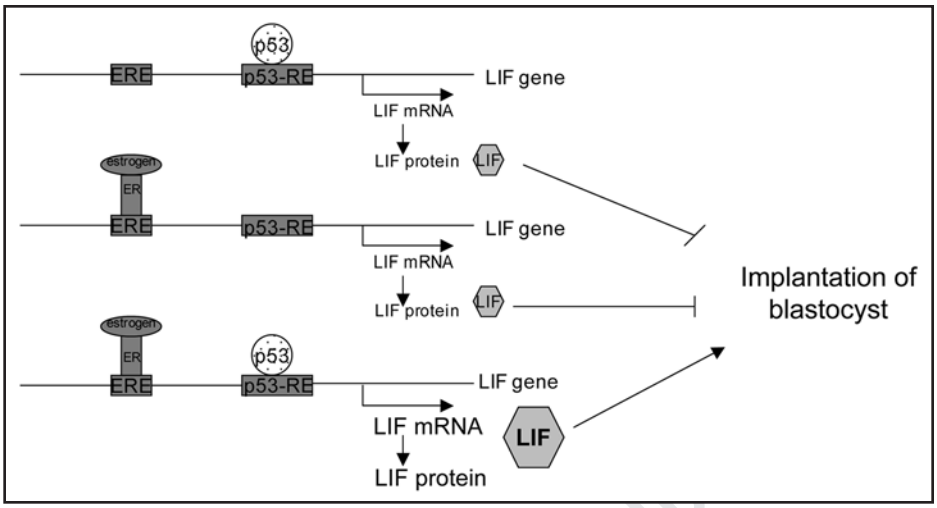

Figure 2. Cooperative regulation by p53, estrogen and estrogen receptor (ER) is required for LIF expression at sufficient level, which is crucial for implantation of blastocysts. Expression of the LIF gene can be regulated by p53 through p53 binding element (p53 RE) (upper), as well as by estrogen and ER through estrogen responsive element (ERE) (middle). Cooperative regulation by both p53 and estrogen, ER at the onset of implantation is required to induce LIF expression at sufficient level, which is crucial for implantation of blastocysts (lower).

In humans, implantation is also a pivotal event in pregnancy. It has been reported that implantation is relatively inefficient in humans, and implantation failure is the most frequent cause of lack of pregnancy after in vitro fertilization (IVF) and embryo transfer.[45] The presence of a sufficient amount of human LIF protein in the uterus at the time of implantation has been suggested as an essential condition for implantation.[46] It is possible that p53 plays a similar role in human fecundity through regulation of uterine LIF levels at the implantation stage. Indeed, a study from Carolyn Coulum's group reported that the P72 allele of the p53 gene, which has an altered p53 transcriptional activity compared to the R72, is associated with the recurrent implantation failure in an IVF clinic.[47] While its mechanism is still unclear, it is possible that the P72 SNP may alter the activity of p53, and in turn result in the reduced uterine LIF levels and decreased implantation rates in this group of women. It will be of interest to study whether this group of women with a P72 allele in the p53 gene has lower LIF levels in their uteri. Thus an increased effectiveness of the R72 allele in implantation and its decreased impact upon longevity would be a classic example of antagonistic pleiotrophy.

This study has uncovered novel functions for the p53 gene in mice and likely in humans. The interconnections between faithful development, stress management, protection from cancers and longevity appear to connect to a central integrator protein: p53. Each of these four processes demonstrates sexually dimorphic phenotypes and both the LIF gene and the Mdm2 gene are co-regulated by p53 and estrogen, giving rise to these sexually dimorphic properties. The next few years will uncover additional roles for the p53 network in metabolism, the nervous system, the immune system and possibly other diseases. The p53 pathway will not only integrate molecular events in a cell but systemic events between organ systems in an organism.

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