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Review

Li–Fraumeni Syndrome Disease Model: A Platform to Develop Precision Cancer Therapy Targeting Oncogenic p53

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Li–Fraumeni syndrome (LFS) is a rare hereditary autosomal dominant cancer disorder. Germline mutations in *TP53*, the gene encoding p53, are responsible for most cases of LFS. *TP53* is also the most commonly mutated gene in human cancers. Because inhibition of mutant p53 is considered to be a promising therapeutic strategy to treat these diseases, LFS provides a perfect genetic model to study p53 mutation-associated malignancies as well as to screen potential compounds targeting oncogenic p53. In this review we briefly summarize the biology of LFS and current understanding of the oncogenic functions of mutant p53 in cancer development. We discuss the strengths and limitations of current LFS disease models, and touch on existing compounds targeting oncogenic p53 and *in vitro* clinical trials to develop new ones. Finally, we discuss how recently developed methodologies can be integrated into the LFS induced pluripotent stem cell (iPSC) platform to develop precision cancer therapy.

Trends

LFS is a cancer hereditary syndrome caused by *TP53* germline mutations. This syndrome serves as a useful model to study mutant p53-associated cancers. LFS patient-derived iPSCs offer several advantages compared to other LFS disease models, including unlimited supply of tissue, a human platform, and access to the heterogeneity of disease across multiple cell types. This system enables cancer modeling and facilitates *in vitro* drug testing.

LFS iPSCs and engineered p53 mutant PSCs can be used to discover drugs that target mutant p53 and its related pathways. LFS iPSCs can also assist in the development of *in vitro* assays, which are of great value in drug screening and testing in a dish. Marriage of LFS iPSC models to precise genome editing, 3D-based cell culture, and organ-on-chip systems will facilitate cancer modeling and anticancer drug discovery in a more comprehensive and nuanced way than would otherwise be possible.

Discovery of LFS Syndrome and Identification of *TP53* As a Crucial Gene for Tumorigenesis

LFS [Online Mendelian Inheritance in Man (OMIM) 151623; https://www.omim.org/entry/151623] is a rare familial autosomal dominant cancer syndrome characterized by early onset of multiple tumors, particularly soft-tissue sarcomas, osteosarcomas, breast cancers, brain tumors, adrenocortical carcinomas, and leukemia. LFS was first described in 1969 by Li and Fraumeni [1,2] (Figure 1). In reviewing 280 medical charts and 418 death certificates of 648 childhood rhabdomyosarcoma patients in the USA from 1960 to 1964, they identified four families in whom a second child had developed a soft tissue sarcoma. These four families also had striking histories of breast cancer and other neoplasms, suggesting a previously undescribed familial cancer syndrome.

The classic LFS pedigree was defined in the proband as a patient with sarcoma diagnosed before age 45 years, plus a first-degree relative with any cancer before age 45 and another first- or second-degree relative with any cancer before age 45 or a sarcoma at any age. This definition was based on 24 kindreds with the syndrome of sarcoma, breast carcinoma, and other neoplasms in young patients [3]. The defined criteria for this syndrome gradually evolved

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to include not only classic LFS but also LFS-like syndrome (LFL), which shares features of LFS but does not conform to the strict definition [4].

While the six core cancers mentioned above account for the majority of LFS-associated tumors, the remaining cancers include diverse carcinomas of the lung, stomach, ovary, and colon/rectum, as well as lymphoma, melanoma, and other neoplasms [5]. One half of patients with LFS develop at least one LFS-associated cancer before age 30, compared to a 1% incidence of cancer before age 30 in the general population [6]. The lifetime risk of cancer in LFS is estimated to be 73% for males and almost 100% for females, with the increased risk of breast cancer accounting for the difference [7,8].

LFS patients are also at a remarkably increased risk of developing a second malignancy [9,10]. A study of 200 LFS patients from 24 kindreds showed that 57% of patients developed a second malignancy within 30 years after diagnosis of the first cancer [10]. LFS patients also are at increased risk of developing treatment-related secondary malignancies. Several case reports suggested that ionizing radiation-induced cancers are more common in LFS patients [11–13], and research studies also support this relationship [14,15]. Therefore, radiation therapy is generally avoided in the management of these patients if possible. A stringent surveillance strategy is one of the key components of LFS patient management. A prospective clinical trial aimed at improving cancer screening for LFS patients showed that a surveillance strategy including whole-body magnetic resonance imaging (MRI) and other biochemical tests was able to detect tumors earlier, and this was associated with improved long-term survival [16].

In 1990, Malkin *et al*. used a candidate gene approach to first link a *TP53* germline mutation to LFS [17]. Srivastava *et al*. later analyzed *TP53* mutations in a LFS family, identifying the same point mutation in codon 245 of the *TP53* gene in different generations of this pedigree [18]. Together with the previous observations that p53 is also inactivated in the sporadic (non-familial) forms of cancers, these studies suggested that loss of p53 is a rate-limiting step for tumorigenesis [17], and implied that inherited *TP53* mutations could be responsible for the increased susceptibility to cancer.

The initial detection of a *TP53* heterozygous point mutation was only in five LFS families [17]. Numerous subsequent studies have shown that ∼70% of LFS families harbor detectable germline *TP53* mutations [4,19–22]. These mutations are also highly associated with a significant increase in DNA copy-number variations (CNVs) [23]. For those patients without detectable *TP53* mutations, a complete heterozygous germline deletion of *TP53* was reported [24], indicating that rearrangements affecting *TP53* occur rarely, but should be considered in LFS families. According to the National Comprehensive Cancer Network (NCCN) guidelines (https://www.nccn.org/), *TP53* genetic testing is recommended for individuals from a family with a known *TP53* mutation, or for individuals who meet either the classic LFS criteria, the Chompret criteria [25], or who were diagnosed with breast cancer before age 31. To date, *TP53* germline variants from more than 700 LFS pedigrees have been reported and integrated into the International Agency for Research on Cancer (IARC) TP53 Database (http://p53.iarc.fr/) that provides updated resources of hereditary *TP53* variants, including the distribution of mutations over the *TP53* gene and the influence of different types of mutations on the tumor spectrum [26].

Biological Functions of p53: Ever-Growing Complexity

As a central regulator within an extremely complex biological network, p53 plays a much broader role in many normal cellular and developmental processes in addition to its well-known tumor-suppressor function. The most extensively studied mechanism by which wild-type (WT)

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p53 functions is as a transcription factor, regulating the expression of a set of target genes involved in cell-cycle arrest, senescence, apoptosis, and DNA damage repair [27–29]. Beyond these classic functions, p53 also plays roles in regulating glucose metabolism [30,31], antioxidant activity [31–33], autophagy [34,35], motility and invasion [36], angiogenesis [37], bone remodeling [38,39], stem cell self-renewal [40,41], differentiation [42–49], and somatic cell reprogramming [50–52]. Most of these biological processes regulated by p53 have also been proved to contribute to its tumor-suppressive effect [29], although there is emerging evidence of a 'dark side' of p53 in which WT function can prevent cell death or even promote cancer development and progression [53–55].

Although much still needs to be understood about this ever-growing p53-associated network, it is clear that restoration of functional WT p53 leads to tumor growth suppression [56–58]. Therapeutic manipulation of p53 is an ongoing hot research field, and more than 100 related clinical trials have been conducted [59]. Translation of our current knowledge of p53 into the clinic has been challenging and is often complicated by the interplay between WT and mutant p53, **mutant p53 gain-of-function** (see Glossary), inconsistent findings from different study systems, and the versatility of p53 in distinct microenvironments. Even so, novel and creative approaches to treat p53-associated diseases are in high demand.

Mutant p53 Gain-of-Function: More Than Merely a Loss

TP53 is the most frequently mutated gene in human cancers [60,61]. Deletion or truncation mutations in p53 abrogate its normal function by attenuating p53-responsive cellular activities; when both alleles become mutated, the anticancer protection of p53 is shut down. Although **p53 loss of function** is a common feature of cancer, p53 does not fully follow the classic **Knudson's two-hit theory** during carcinogenesis or cancer progression [62]. It is not simply the loss of WT p53 that drives cells to cancer. The majority of the p53 mutations found in tumors are missense mutations, resulting in production of full-length protein with only a single amino acid change [63]. Many of these mutant proteins exhibit a **mutant p53 dominant negative effect** over WT p53, mostly by forming mixed tetramers with diminished DNA-binding and transactivation activity [64]. In this way, mutation of only one copy of p53 can lead to many of the downstream effects that would otherwise require loss of both copies.

Probably the most striking fact about the p53 mutation landscape in cancer is the high prevalence of missense substitutions at particular locations, mainly in the DNA-binding domain (DBD) [63] (Figure 2). These 'hotspot' mutations indicate selective advantages during cancer development and progression. Indeed, many hotspot mutations arm the mutant p53 with new weapons to promote cancer. Such activities, known as mutant p53 gain-of-function, are involved in the regulation of various cancer hallmarks (Figure 3), including genomic instability [65–69], anti-apoptotic activities [70–79], replicative mortality [69,80], invasion and metastasis [63,64,66,67,79,81–91], angiogenesis [92–95], dysregulated metabolism [96–99], and tumor-related inflammation [100–103]. Mutant p53 gain-of-function can drive cancer through several potential mechanisms [104,105]: (i) binding to structure-specific DNA to subsequently exert transcriptional regulation, (ii) interacting with transcription factors or cofactors to enhance or decrease the transcription of their targeted genes, (iii) associating with chromatin or the chromatin regulatory complex, and (iv) directly interacting with and influencing other proteins and their functions.

Several excellent reviews [64,104–113] have addressed p53 gain-of-function from various aspects. We highlight here some of the most recent discoveries on mutant p53 gain-of-function and address their therapeutic potential in cancer treatment.

Glossary

Conditional reprogramming (CR): a cell culture methodology that is applied to rapidly and efficiently establish cells for long-term propagation using normal and cancer cells taken directly from patients.

HUPKI mouse model: the human *TP53* knock-in mouse model is constructed using gene-targeting technology to generate a mouse strain harboring the human wild-type (WT) *TP53* genome in both copies of the mouse *Tp53* gene locus. *Tp53* exons 4–9 are replaced with the human gene *TP53* exons 4–9.

Induced pluripotent stem cells (iPSCs): pluripotent stem cells derived from differentiated somatic cells through somatic reprogramming by defined factors (e.g., OCT4, SOX2, KLF4, and c-MYC).

Knudson's two-hit theory: Alfred G. Knudson formulated this theory in 1971 by analyzing cases of retinoblastoma which occur as autosomal dominant inherited disease and sporadically. The fact that inherited retinoblastoma occurs at a younger age than the sporadic case, and tumors often affect both eyes, led to Knudson's hypothesis: the first hit is present in the germline, but the first hit (germline mutation) at the susceptibility locus is not sufficient for tumor formation; a second hit (somatic mutation) is necessary for tumor formation. Knudson's 'two-hit' theory led to the identification of *RB1* as a tumor-suppressor gene and also provides evidence for other tumor-suppressive genes such as *WT1*, *APC*, and *TP53*.

Mutant p53 dominant negative effect: some p53 mutants, when expressed together with WT p53, can inactivate WT p53 and overcome its tumor-suppressive functions. This dominant negative effect of mutant p53 over WT p53 protein can be achieved by forming mixed tetramers with diminished transactivation activity, therefore promoting tumorigenesis.

Mutant p53 gain-of-function: specific p53 mutants possess functions of their own that are entirely independent of those observed in WT p53. These newly gained functions are usually oncogenic, contributing to cancer progression from various aspects.

Regulating Imprinted Genes

Aberrant imprinting or dysregulation of imprinted genes is associated with developmental disorders and an increased risk of cancer [114–116]. Alterations in the expression of genes in the imprinted *H19–IGF2* locus have been described in Beckwith–Wiedemann syndrome (BWS) [117] and Russell–Silver syndrome (RSS) [118], which are associated with risk for Wilms tumor, hepatoblastoma, and rhabdomyosarcoma. Bidirectional links between WT p53 and imprinted genes in the *H19–IGF2* locus have been demonstrated in several studies [119–121]. Loss of imprinting of *IGF2* accelerates tumor formation by inactivating WT p53 [119]. Maternally imprinted H19, which encodes a long noncoding RNA, has been shown to be negatively regulated by p53 [120]. In 2015, this link was extended to the mutant p53. A study by Lee *et al*. revealed that numerous p53 mutants exhibit gain of function and can inhibit H19 expression [122]. Using LFS patient-derived **induced pluripotent stem cells** (iPSCs) to model osteosarcoma, this study found that the p53 (G245D) mutant represses H19 expression during osteoblastic differentiation. Moreover, many hotspot p53 mutants including R175H, G245S, R248W, and R280K also showed strong inhibition of H19 expression compared to WT p53, indicating that mutant p53 gain-of-function in regulating imprinted gene expression is a general mechanism in LFS-associated osteosarcoma across distinct p53 mutations.

Driving Cancer through Interplay with Chromatin

One of the features of gain-of-function mutant p53 is the ability to associate with chromatin and other transcriptional factors to globally influence the gene expression profile [77,96,101,123,124]. Recently, it was shown that multiple mutant p53 forms bind to the SWI/SNF chromatin remodeling complex [95]. This interaction with the SWI/SNF complex mediates up to 40% of the mutant p53 regulated genes, including the angiogenesis-promoting gene *VEGFR2*, further suggesting that repressing the SWI/SNF complex or its downstream targets (e.g., by anti-VEGF) may help to reverse the changes caused by mutant p53 in cancer. A proteome-wide analysis found that the p53 R273H mutant is tightly associated with chromatin and can modulate the protein levels of PARP, PCNA, and MCM4 in a transcription-independent manner [125]. Inhibition of PARP activity showed efficacy in treating these mutant p53 expressing cancer cells. Another mutant p53 gain of function causes mutant p53 to interact specifically with transcription factor ETS2 and leads to upregulation of chromatin regulatory genes, including *MLL1*, *MLL2*, and *KAT6A*, resulting in a global increase of histone modifications (methylation and acetylation) and tumor progression [126]. This suggests the possibility that therapeutic inhibition of the MLL1 methyltransferase complex might decrease cancer cell proliferation.

Driving Oncogene Expression by Repressing Transcription Factors

Mutant p53 can exert its pro-oncogenic properties by physically interacting with the p53 family members, p63 and p73, and altering their transcriptional activity [78,81,127,128]. Mutant p53 promotes pancreatic cancer invasion and metastasis by upregulating the cancer driver PDGFRB [129]. p53 mutants at the hotspot sites R175H and R273H were shown to bind to the p73/NF-Y complex. This interaction impairs the repressive transcriptional regulation of p73 at the *PDGFRB* promoter region. The study also showed that inhibition of PDGFRB using RNAi or small molecules is effective in attenuating metastasis *in vivo*, suggesting a possible target in controlling metastasis in p53 mutant pancreatic cancer.

Current LFS Disease Models

Engineered Mouse Models

Engineered mouse models have been used extensively for mammalian *in vivo* and *in vitro* studies of LFS. In 1992, to investigate the role of the *Tp53* gene in mammalian development and tumorigenesis, a null mutation was introduced into the gene by homologous recombination in murine embryonic stem cells [130]. Mice homologous for the null allele ($Tp53^{-/-}$) appear to be

Organoid: a 3D culture system in which a collection of multiple organ-specific cells self-organize into organ-bud structures. 3D-cultured organoids mimic better the microanatomy of organs and are capable of recapitulating specific organ functions, enabling experimental study of otherwise inaccessible tissue.
Organs-on-chip: a microfluidic device containing one or more culture chambers connected with channels allowing exchange of culture media. The cells cultured in each chamber can proliferate, differentiate, and mature in a more physiological environment, and mimic the smallest functional subunits of a human tissue or organ. Incorporation of human iPSCs, 3D culture, and vasculature in the microfluidic device makes organs-on-chip a promising platform for drug screening and discovery.
p53 loss of function: WT p53 can exert a tumor-suppressive effect by regulating cellular functions such as cell-cycle arrest, apoptosis, and DNA damage repair. When the *TP53* gene undergoes mutations, the tumor-suppressive effect of WT p53 can be abrogated or completely lost, which contributes to the course of tumor development.
Patient-derived tumor xenograft (PDTX): a xenograft model created when surgically resected tumor samples from patients are engrafted directly into immunodeficient mice. Tumors in the PDTX model can be maintained through serial passaging in mice. PDTXs recapitulate the molecular, genetic, and histological characteristics of the primary tumors of origin; they therefore offer an excellent *in vivo* preclinical platform for novel cancer therapeutics discovery.

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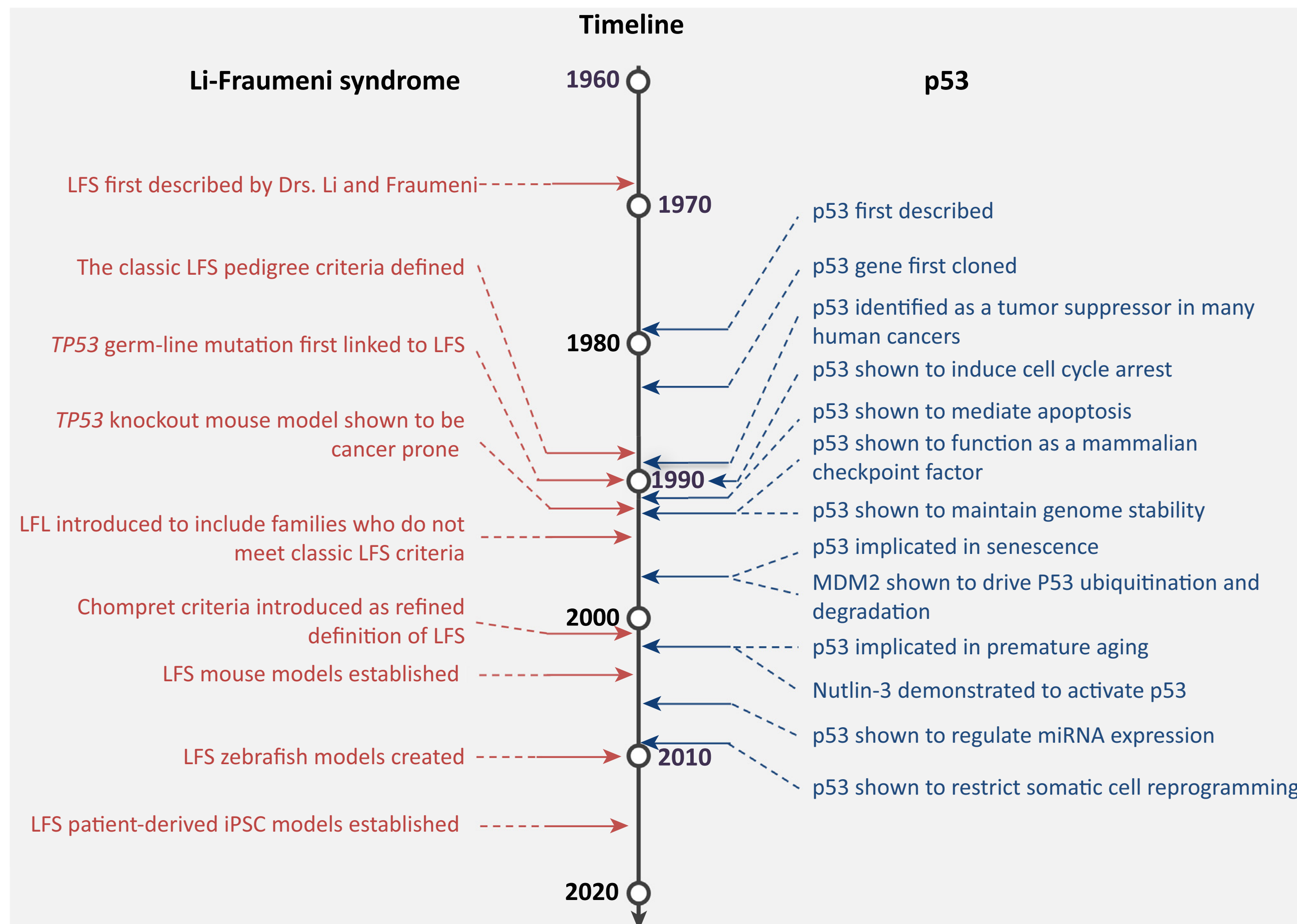

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Figure 1. Milestones of Li–Fraumeni Syndrome (LFS) and p53 Research. The left column timeline shows important research developments in LFS, including discovery of the disease, identification of the underlying genetic cause, and establishment of a disease model. The right column timeline lists the equivalent key findings during 38 years of research on p53. Abbreviations: iPSC, induced pluripotent stem cell.

developmentally normal but are highly susceptible to early onset of a variety of neoplasms. Subsequent homozygous knockout mice with different deletions of *Tp53* showed similar tumorigenic phenotypes [131,132].

However, a more appropriate genetic model for LFS with heterozygous mutations in *Tp53* is needed because LFS patients are invariably heterozygous rather than homozygous for mutant p53. With this in mind, heterozygous p53-null mice ($Tp53^{+/-}$) were generated. Nearly 50% of them developed tumors by 18 months of age, although with a comparative cancer onset delay compared to the homozygous mice. This was roughly similar to the 50% cancer incidence before age 30 in affected LFS individuals, given that C57BL/6 mice have an average lifespan of 30 to 36 months [133]. The similarities between $Tp53^{+/-}$ mice and LFS patients are even more striking with respect to their tumor spectrum. $Tp53^{+/-}$ mice develop osteosarcomas and soft tissue sarcomas, as seen with high frequency in LFS families, while homozygous mice predominantly develop malignant lymphomas [134].

Missense mutations are the most common mutations in affected LFS individuals apart from null mutations. Mouse models of these mutations garnered significant attention in the p53 research

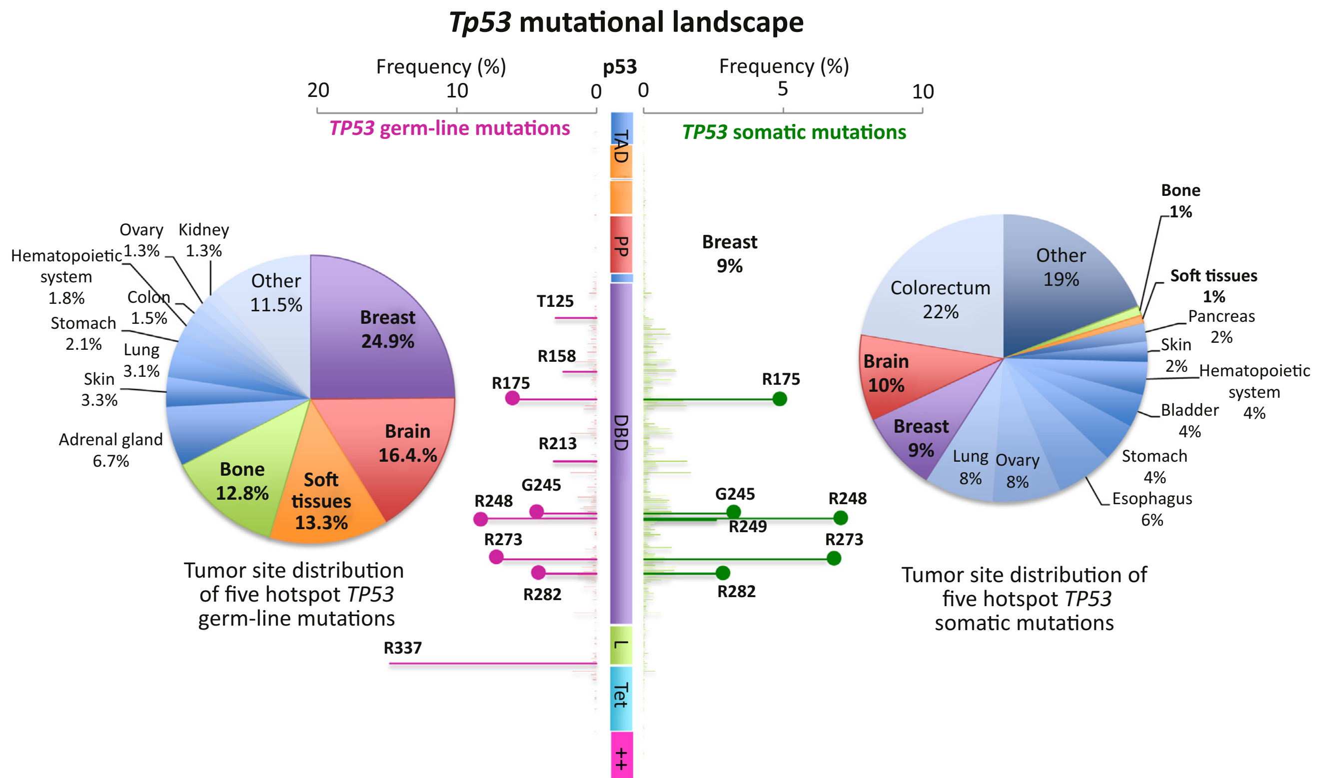

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Figure 2. Mutational Landscape of *TP53* Germline and Somatic Mutations in Human Cancer. *TP53* missense mutation data are obtained from the International Agency for Research on Cancer (IARC) TP53 database (http://p53.iarc.fr/). The distribution of p53 mutations is plotted over the function of amino acid position; the left side indicates germline mutations and the right side indicates somatic mutations. The horizontal axis shows the frequency of any mutation at the indicated residues. The vertical axis represents p53 protein starting with the N-terminus at the top. p53 protein contains transcriptional activation domains I and II (TAD 1, 20–40; TAD II, 40–60), the proline domain (PP, 60–90), the sequence-specific core DNA-binding domain (DNA-binding core, DBD; residues 100–300), the linker region (L, 301–324), the tetramerization domain (Tet, 325–356), and the lysine-rich basic C-terminal domain (++, 363–393). The most common mutations or hotspots are indicated in bold; residues R175, G245, R248, R273, and R282 are the five common hotspots for both germline and somatic mutations (indicated as a lollipop). Pie charts illustrate the tumor site distribution of five hotspot *TP53* mutations (left, germline; right, somatic). Malignancies of breast, brain, soft tissues, and bone are the most commonly seen for the five hotspot germline mutations; malignancies from these tumor sites are also distributed in the same five hotspots of *TP53* somatic mutations (indicated in bold).

community. In 2000 the first heterozygous mouse containing an R to H substitution at p53 amino acid 172 was generated, which corresponds to the R175H hotspot mutation in human cancers and the germline mutation in LFS kindreds [135]. Although this model contained an unexpected deletion of a G nucleotide at a splice junction that attenuated levels of mutant p53 to near WT levels, mice heterozygous for the mutant allele differed from $Tp53^{+/-}$ mice because the osteosarcomas and carcinomas developed in these missense mutant mice frequently metastasized (69% and 40%, respectively). This indicated, for the first time, that a p53 missense mutation could confer a gain of function *in vivo*, even when expressed at relatively low levels.

Later in 2004, two groups independently reported knock-in mouse models of LFS expressing the p53 mutant alleles R172H and R270H ($Tp53^{M/-}$), equivalent to the codons 175 and 273 in humans [136,137] (Table 1). Both studies demonstrated that $Tp53^{M/-}$ mice developed a broader tumor spectrum with a more invasive and metastatic phenotype compared to $Tp53^{+/-}$ mice, although no change in survival was observed. The broad spectrum of tumors included a variety of carcinomas, bone sarcomas, leukemias, and even a glioblastoma

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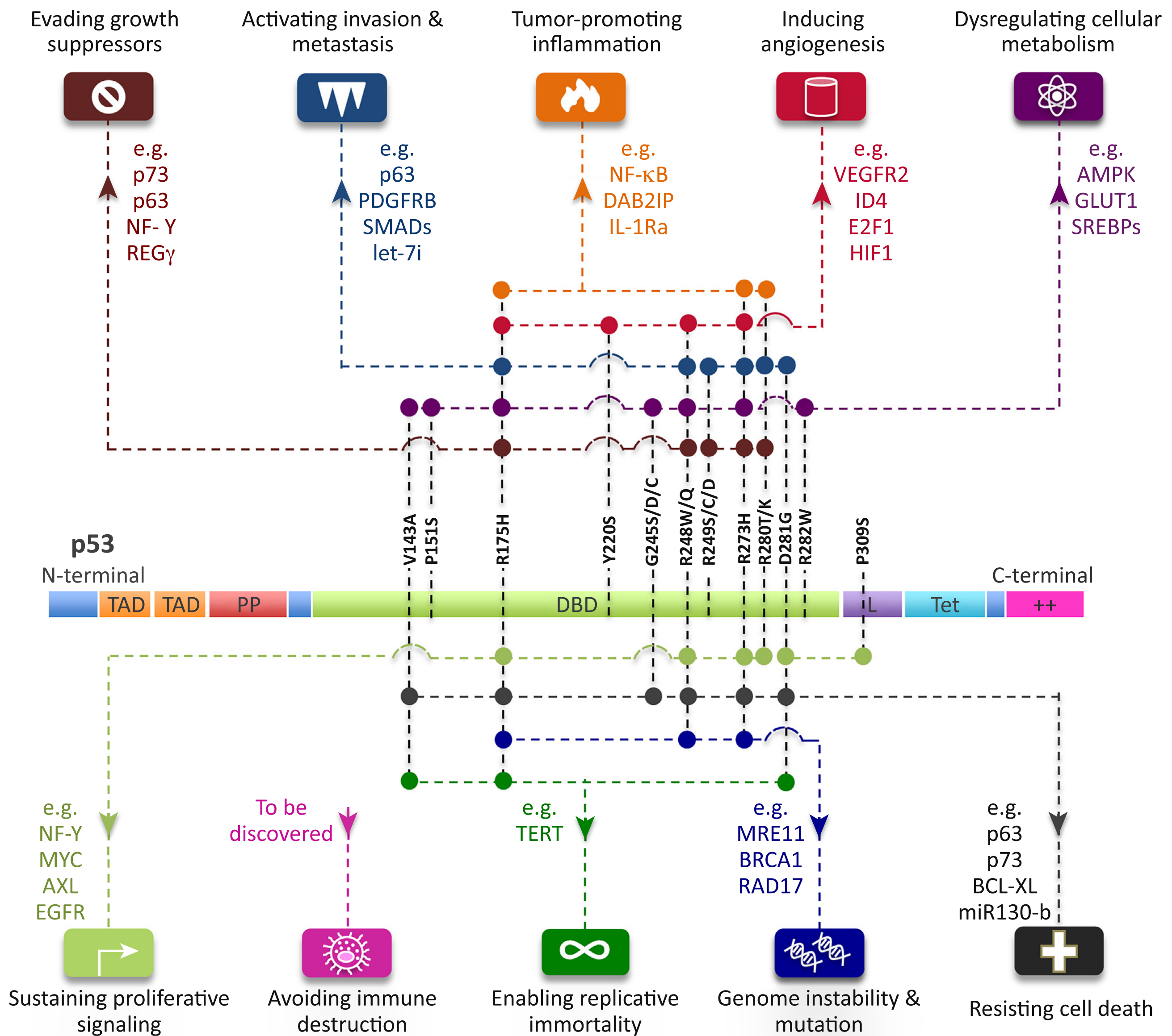

Figure 3. Mutant p53 Gain-of-Function Cancer Driver Mutations and Hallmarks of Cancer. Different mutations in p53 protein (structural domains are described in Figure 2) arm p53 with new weapons (downstream targets indicted in the figure) to drive cancer development and progression. Each color-coded node indicates gain-of-function of a specific mutation in *TP53* which further drives cancer through various hallmark properties of cancer cells.

Table 1. Current LFS Disease Models[a]

Model	p53 mutation	Mutant p53 function	LOH	Tumor types	Metastasis	Model system	Refs
Zebrafish	I166T	Dominant negative	Yes	Broad spectrum	N.A.	*In vivo*	[141]
Heterozygous mouse	R172H and R270H	Gain of function	Yes	Broad spectrum	Yes	*In vivo*	[135,136]
HUPKI mouse	R175H, R245S, R248Q, R248W, and R273H	Gain of function	N.A.	Broad spectrum	N.A.	*In vivo*	[65,138,139]
Patient-derived iPSCs	G245D	Gain of function	N.A.	Osteosarcoma	N.A.	*In vitro*	[122]

[a]Abbreviations: LOH, loss of heterozygosity; N.A., not available.

multiforme (GBM, the most common brain tumor in LFS), indicating that $Tp53^{M/-}$ mice better recapitulate the human LFS familial syndrome than do $Tp53^{+/-}$ mice. Interestingly, $Tp53^{M/-}$ and $Tp53^{+/-}$ mice did not develop breast cancer, one of the most common tumors in LFS patients, but increased the incidence of hematological malignancies (e.g., lymphomas) [136,137], implying that either species differences or a specific susceptible genetic background influence the ability of these model models to recapitulate human LFS.

Similarly, **HUPKI mouse models** (humanized *TP53* knock-in models) [138] were constructed by targeting the mutant human *TP53* DNA sequence into murine embryonic stem cells (Table 1). HUPKI models were generated for the human mutations R175H, G245S, R248W, R248Q, and R273H [65,139,140]. All these knock-in mice except the G245S model showed a broader tumor spectrum than *Tp53* null mice, providing strong support for the gain-of-function hypothesis of various missense p53 mutants in driving and enhancing spontaneous tumorigenesis.

Zebrafish Models

Another model of LFS was created in zebrafish (Table 1), a powerful vertebrate system that is accessible to both large-scale screens and *in vivo* manipulation for cancer studies [141]. A forward genetic screen was performed using a specific ionizing radiation (IR)-induced phenotype in zebrafish embryos, leading to the identification of the p53 I166T mutations. This mutation was shown to give rise to tumors, predominately sarcomas, with 100% penetrance in adult fish. As in humans with LFS, heterozygous $tp53^{I166T}$ fish follow Knudson's two-hit hypothesis, and the tumors displayed loss of heterozygosity (LOH) at the *tp53* locus. Additionally, the data demonstrated that the p53 regulatory pathway, including Mdm2, is evolutionarily conserved in zebrafish. This work demonstrated the potential of zebrafish models to discover novel genes and therapeutic compounds that modulate the evolutionarily conserved LFS pathway.

Primary Cell Line Systems

Researchers have also gained insight into LFS through direct investigation of patient primary cells. A comparison of soft tissue sarcomas, including fibrosarcomas, from affected LFS patients with fibroblasts derived from skin biopsies from the same patients demonstrated chromosomal anomalies, resistance to senescence, and spontaneous immortalization in the LFS fibroblasts compared to control cultures [142,143]. Immortalization of these cells appeared to be associated with loss of the WT *TP53* allele, $p16^{INK4A}$ (*CDKN2A*) expression, and telomere elongation [144–146]. Loss of p53 during this immortalization has been shown to cause a decrease in TSP-1 expression, a potent inhibitor of angiogenesis, and switch the LFS fibroblasts to a pro-angiogenic phenotype [147,148]. In addition, normal breast epithelial cells obtained from a patient with LFS (with a mutation at codon 133 of the *TP53* gene) spontaneously immortalized during *in vitro* culture, while breast stromal fibroblasts from this same patient did not [149]. The immortalization of normal cells from LFS patients strongly indicates that transformation is characteristic of the LFS genetic background.

Patient-Derived iPSCs

The motivation for use of patient-derived iPSCs stems from limitations inherent to other systems. Animal models do not fully represent human LFS disease features, while primary cells from affected patients are limited to a few cell types [150,151]. To access a wider spectrum of cell types, iPSCs were generated from patient fibroblasts obtained from a LFS family with a heterozygous *TP53*(G245D) hotspot mutation and differentiated into targeted lineages [122] (Table 1). Despite their defective p53 function, LFS iPSC-derived mesenchymal stem cells (MSCs) maintained normal MSC characteristics and could be differentiated into osteoblasts. Interestingly, once turned into osteoblasts, genome-wide transcriptome analysis revealed that the cells expressed an osteosarcoma signature. LFS osteoblasts recapitulated the differentiation defects and oncogenic properties of osteosarcoma. Part of this phenotype

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was shown to be mediated by repression of the imprinted gene *H19* by mutant p53 gain-of-function. Furthermore, LFS osteoblasts and tumors in this model system showed a negligible number of the cytogenetic rearrangements that are commonly found in osteosarcoma, indicating the existence of a relatively intact genome in this model system and the feasibility of studying early cancer progression before the accumulation of broad genome alterations. These data show that the LFS iPSC disease model successfully transforms clinical samples into cell line models. As techniques for directed differentiation improve, this technique may be applied to study many more cancer types in affected LFS families.

Prospective LFS Disease Models

Existing LFS models have several limitations. Mouse and zebrafish models have species differences from human LFS patients. Primary cells from LFS families are limited to only a few cell types and are challenging to obtain and maintain. Only a limited number of adult tissue types are currently accessible through directed differentiation of LFS iPSCs. The developing **patient-derived tumor xenograft** (PDTX) and **conditional reprogramming** (CR) methods may provide alternative approaches to compensate for the limitations of current models.

PDTXs are obtained by directly implanting freshly resected patient tumor pieces subcutaneously or orthotopically into immunocompromised mice [152]. Numerous tumor-specific PDTX models have been established. Importantly, they are biologically stable when passaged in mice in terms of global gene-expression patterns, mutational status, metastatic potential, drug responsiveness, and tumor architecture [153]. They often preserve the molecular and cellular basis of tumor heterogeneity, and have been shown in some cases to predict therapeutic responses [154]. These features suggest that PDTX models may reliably predict clinical activity of novel compounds in cancer patients.

CR is a cell culture technique that is used to rapidly and efficiently establish patient-derived cell cultures from both human normal and tumor cells [155], which is accomplished by co-culturing them with irradiated mouse fibroblast feeder cells in the presence of a Rho kinase inhibitor (Y-27632). In CR culture conditions, cells are rapidly reprogrammed to cells with the characteristics of adult stem cells. When transferred into conditions that mimic *in vivo* environments, they reverse back to differentiated states and organize into structures similar to the tissue from which they were derived. Compared to immortalized cells, CR cells maintain the phenotypic and genotypic features of the primary tumors, thus providing a faithful preclinical model. Moreover, taking advantage of its rapid and efficient expansion of cell cultures, CR can be applied to drug screening sufficiently quickly to provide information for clinical use. In addition, the large amounts of cells generated can be used in PDTX and **organoid** cultures.

In conclusion, the combination of the techniques mentioned above provides a brighter vision of the disease modeling (Figure 4).

Translating LFS iPSC Models into Clinical Therapies

Preclinical Trials

Animal models are conventionally used to test the efficacy and toxicity of preclinical compounds. Although these surrogate models are valued as a gatekeeper for clinical trials, they often yield disappointing results because of fundamental differences between species [156,157]. Failure to translate results from animal models into clinical trials led to suggestions that therapies could also be tested for efficacy on specimens collected from patients during preclinical trials. This concept of *in vitro* clinical trials, as advanced by the FDA, also emphasizes the use of new scientific methodology that enables testing treatment strategies on living human tissues [158]. The availability of iPSC technology has augmented the potential of clinical trials in a dish [157]. Increasingly refined differentiation protocols have enabled the generation of large

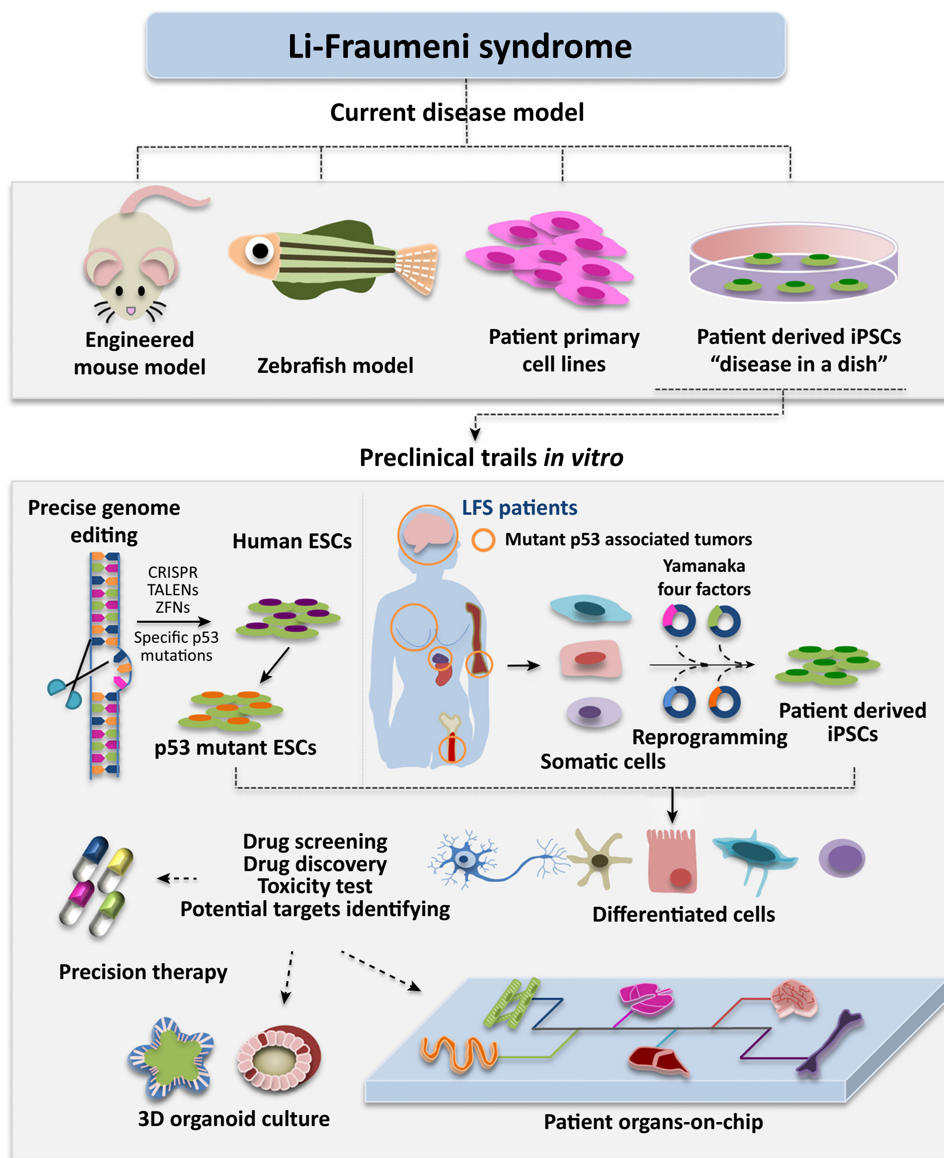

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Figure 4. Application of LFS iPSC Models to Drug Development for LFS and p53 Mutation-Associated Tumors. The LFS iPSC model overcomes the limitations of current LFS disease models such as those based on mice, zebrafish, or primary cell lines, and holds potential in modeling LFS-associated cancers and facilitating preclinical trials. Precise genome-editing techniques make it possible to expand the bank of PSCs with different p53 mutations, which provides a valuable resource for precision cancer medicine. Integration of 3D organoid and organs-on-chip systems with an LFS iPSC disease model offers exciting opportunities for testing existing both WT and mutant p53-associated pathway-related drugs and discovering new therapeutic compounds. Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells, LFS, Li–Fraumeni syndrome; PSC, pluripotent stem cells; WT, wild type.

quantities of differentiated cells of various types from patient-derived iPSCs. An unlimited supply of otherwise inaccessible disease-relevant cells should permit *in vitro* drug screening, toxicity testing, and drug response prediction.

Patient-derived iPSCs have been used to model various diseases, including long-QT syndrome (LQTS) [159,160], α1 antitrypsin (AAT) deficiency [161,162], familial dysautonomia (FD)

[163,164], Diamond–Blackfan anemia (DBA) [165–167], familial Alzheimer's disease [168], and RASopathy disorders [169,170], to name a few. Successful disease modeling not only sheds light on disease mechanisms but also leads to the development of *in vitro* assays – readouts of disease-associated phenotype – that facilitate high-throughput drug screening. AAT-deficiency patient iPSC-derived hepatocyte-like cells have been used to conduct large-scale screening of clinically available compounds for the purpose of discovering potential treatments [171]. High-throughput screening to identify small-molecule compounds to rescue IKBKAP expression in FD iPSC-derived neural crest cells revealed the potential of an α2-adrenergic receptor (α2-AR) antagonist in FD treatment [163]. Another recent study also utilized iPSC-derived hematopoietic progenitors to perform a non-biased drug screening for DBA, and identified autophagy as a therapeutic pathway in this rare blood disorder. iPSC-based drug screening has also been applied to diseases other than genetic disorders, such as infectious diseases. iPSC-derived hepatocytes have been used to screen drugs for treating chronic infectious diseases such as hepatitis B and liver-stage malaria [172,173]. High-throughput drug screening has also been carried out to identify potential antiviral drugs using iPSC-derived cardiomyocytes and neurons from patients with viral cardiomyopathy or Zika virus infection [174,175].

One of the crucial steps in developing clinical drugs is to test toxicity, and this often leads to failure and/or withdrawal of preclinical drugs [176]. Increasing attention is being paid to models for predicting drug-induced toxicity at the single-patient level. Human iPSC-derived cardiomyocytes (iPSC-CMs) are proving useful in predicting a drug-induced prolonged action potential (also referred to as long QT) which places patients at high risk of life-threatening cardiac arrhythmias [177]. A recent study reported the use of iPSC-CMs in predicting doxorubicin-induced cardiotoxicity in cancer patients [178], suggesting the benefits of generating patient iPSCs and examining toxicity before initiating chemotherapy.

LFS iPSC Model: New Opportunities for Screening Compounds

Given its role as a tumor suppressor and its high rate of mutation in cancer, p53 poses an attractive target for cancer therapy. Many human tumors require loss of WT p53 or gain-of-function of mutant p53 to progress to a fully malignant phenotype. Thus, significant efforts have been devoted to p53-based drug development targeting both WT and mutant forms of p53. These strategies include (i) WT p53 activation, (ii) mutant p53 restoration, (iii) mutant p53 elimination, and (iv) p53 family inhibition, as summarized in Table 2.

WT p53 remains at relatively low intracellular levels predominantly due to ubiquitination by the E3 ligase MDM2 targeting it for rapid degradation [179]. In many cancers the MDM2 proteins are dysregulated and exert an oncogenic function mainly by inhibiting p53 tumor-suppressor activity [180]. As a result, considerable efforts have been made to develop compounds that interfere with the p53–MDM2 interaction, leading to the discovery of nutlin-3, RITA, and MI-219 [181–183]. In addition, a new class of stapled peptides, designed to contain a hydrophobic binding interface that mimics the bound α-helical conformation of p53, have been shown to effectively block p53–MDM2 interactions [184–186]. In addition to regulating WT p53, MDM2 can also regulate the degradation of mutant p53, and loss of MDM2 promotes tumor development in mutant p53 mice [187]. This implies that drugs aimed at activating WT p53 by inhibiting MDM2 will also stabilize mutant p53 with adverse consequences.

Mutant p53 is also an attractive druggable target because mutant p53 protein is expressed at high levels in various tumor types but is generally only expressed at very low levels in normal cells [188]. To accomplish this, two strategies have been attempted: restoration of WT p53 transcriptional activity and depletion of mutant p53. The feasibility of restoration of WT activity in mutant protein stems from observations that loss of WT function introduced by some destabilizing mutations can be rescued by additional point mutations that stabilize the conformation

Table 2. Compounds Targeting WT and Mutant p53

Target	Compound	p53	Mechanism	Testing stage	Refs
WT p53 Activation	RITA	WT	Inhibition of p53 binding	Preclinical	[181]
	Nutlin-3	WT	Inhibition of p53–MDM2 interaction	Phase I	[182]
	MI-219	WT	Inhibition of p53–MDM2 interaction	Preclinical	[183]
Mutant p53 restoration	NSC319726	R175H	Restore WT structure and its transactivation function	Preclinical	[189]
	PhiKan083	Y220C	Raise the melting temperature of mutant p53 and slow down its denaturation rate	Preclinical	[190]
	WR-1065	V272M	Restore the WT conformation of the temperature-sensitive p53 mutant V272M	Phase I	[191]
Mutant p53 elimination	Hsp90 inhibitors: 17-AAG	R175H, L194F, R273H, R280K,	Destroy the mutant p53/HSP90 complex to release mutant p53 for degradation	Phase I/II/III	[193]
	HDAC inhibitors: SAHA	R175H, R280K, V247F/P223L	Inhibition of HDAC6 and disruption of the HDAC6/Hsp90/mutant p53 complex	Phase I/II	[194]
	HMG-CoA reductase inhibitors: statins	R156P, R175G, Y220C	Inhibition of the mevalonate pathway, interfere with the HSP40/DNAJA1/mutant p53 complex	Phase I/II/III	[195]
p53 family inhibition	RETRA	R273H, R248W, G266E, R280K	Increase p73 levels and release p73 from the mutant p53/p73 complex	Preclinical	[237]

of the p53 protein [152]. As a result, a variety of compounds that might restore WT p53 function have been characterized, including NSC319726 [189], PhiKan083 [190], and WR-1065 [191].

Depletion of oncogenic mutant p53 also turns out to be effective. Proper function of mutant p53 depends on interactions with the Hsp90 chaperone complex and HDAC6. The Hsp90/HDAC6 chaperone machine is significantly upregulated in tumors compared to normal tissues, and functions as a major determinant of mutant p53 stabilization [192]. Inhibitors that target Hsp90 or HDAC6 both show positive results in depleting mutant p53 in preclinical trials [193,194]. Repression of the mevalonate pathway by statins, which inhibit HMG-CoA reductase, not only abrogates binding of multiple p53 mutants to DNAJA1 and the HSP40 complex but also increases mutant p53 degradation through interaction and ubiquitination by the co-chaperone C-terminus Hsp70/90 interacting protein (CHIP) E3 ubiquitin ligase [195].

Developments in iPSCs methodologies will likely improve drug discovery for p53-related therapies. Because LFS patient-derived iPSCs would provide a more reliable genetic background for drug efficacy and toxicity screening, the failure rate of translation from animal models to humans can be reduced. Moreover, LFS iPSCs can be differentiated into multiple cell lineages, each of which can serve as a tumor model. As a result, a p53 drug screen can be narrowed down to one specific tumor type, increasing the fidelity of the system and the expected success rate. This approach has already shown promise for other genes. Engineered human embryonic stem cell (ESC)-derived neural progenitor cells (NPCs) and neural stem cells (NSCs) have been used to study and model diffuse intrinsic pontine gliomas (DIPGs) [196] and GBM [197], respectively. Both these studies identified novel potential drugs for brain tumors. As applications of iPSC models to cancer increase, the technology will likely gain increasing importance in developing and guiding cancer treatment [198,238].

Personalizing Cancer Therapy through Precise Genome Editing

Advances in genomics have led to an exponential increase in available cancer genomic data and finally directed us to the gene mutations that drive cancer. In some cases mutations found in only a fraction of cells extracted from a single patient tumor sample can be identified.

Nevertheless, translation of this knowledge into personalized therapy is far from reality. One of the biggest bottlenecks is how to convert knowledge of a specific genomic alteration into a therapeutic assay against which therapies can be targeted [199]. Hundreds of *TP53* mutations have been detected in both germline [26] and sporadic tumors [61], but collecting samples from all these patients and assembling them into a biobank of LFS iPSCs would entail a substantial research endeavor.

Precise genome-editing tools such as zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeat/Cas9 (CRISPR/Cas9) present an alternative way around this bottleneck. These site-specific nucleases (SSNs) have proved their power in facilitating site-directed mutagenesis as well as in correcting mutations in PSCs, and are beginning to revolutionize fields of biomedical research [200–202]. By increasing the diversity of genetic diseases available to study and model, these genome-editing tools are facilitating the discovery of therapeutics.

Specific p53 mutations can be engineered into WT pluripotent stem cells (PSCs; including ESCs and iPSCs) by using genome-editing tools, and can thus provide a wide and varied collection of mutant p53 PSCs against which candidate drugs can be screened and tested. Establishment of such a collection of mutant p53 PSCs will allow the testing of existing compounds that target specific p53 mutants (discussed above) on a wider range of p53 mutants (Figure 3). This collection will also facilitate screening and testing of novel potential therapeutics in a more targeted fashion. Finally, with preclinical data that clearly defines the p53 mutants for which a therapy would be expected to be successful, the cost of conducting clinical trials can be dramatically reduced through better patient stratification.

While iPSC technology offers unique advantages in modeling disease down to the genetic background of a particular patient, this specificity can be a double-edged sword. Genetic diversity between individuals often complicates the interpretation of findings across multiple iPSC lines. On the other hand, genome-edited PSCs, either from well-characterized ESCs or iPSCs from healthy subjects, have proved to be useful in revealing disease-relevant phenotypic differences while minimizing the variability found across patient-derived iPSC lines. For instance, introduction of *KCNH2* mutations into human ESCs [199] or integration of *KCNQ1* and *KCNH2* dominant negative mutations [203] into WT PSCs recapitulates the long-QT syndrome phenotype when the PSCs are differentiated into cardiomyocytes. Deletion of the kidney disease genes *PKD1* or *PKD2* induces cyst formation in a PSC-derived kidney organoid model, recapitulating the human disease phenotype [204]. These successful research examples suggest that PSCs with various *TP53* mutations have great potential in elucidating the pathogenesis of mutant p53 associated cancers, facilitating the identification of potential drug targets for tumors with different p53 mutations.

Intersection of the LFS iPSC Model with New Methodologies: Organoids and Organs-on-Chip

Advances in 3D culture technology allow the generation of organoids from PSCs and adult stem cells (AdSCs). These 3D organoids better mimic the physiologic structure and function of organs than 2D culture and have been used to model normal development as well as human diseases [205,206]. Interestingly, the 3D organoid culture system has been extended to primary cancer culture in which cancer organoids can be generated from primary tumors including colon [207,208], pancreatic [209], and prostate cancers [210]. Both normal and cancer organoids provide a unique platform for drug sensitivity and toxicity testing. Mature proximal tubule cells within iPSCs-derived kidney organoids undergo apoptosis after cisplatin treatment, indicating that kidney organoids could be used to test drug nephrotoxicity [211]. Cystic fibrosis patient-derived rectal organoids have been used to characterize the response to

cystic fibrosis transmembrane conductance regulator (CFTR)-modulating drugs, suggesting that organoids can be prospectively used to identify drug responders [212]. A team from the Netherlands generated a living organoid biobank from colorectal cancer patients and demonstrated the feasibility of high-throughput drug screening while highlighting, as an example of potential personalized therapy, the sensitivity of one line to alterations in Wnt signaling [208].

Carcinogenesis and cancer progression can also be modeled using organoids. Introducing mutations of the tumor suppressors *APC*, *SMAD4*, and *TP53*, and the oncogene *KRAS*, into normal intestinal organoids led to malignant transformation both *in vitro* and *in vivo* [213,214]. Neoplastic transformation was also observed when expressing mutant *KRAS* and/or *TP53* in normal PSC-derived pancreatic organoids [215]. Knocking down *Tgfbr2* in *Tp53*$^{-/-}$*Cdh1*$^{-/-}$ murine stomach organoids resulted in a metastatic phenotype *in vivo* [216]. The classic 'adenoma to carcinoma' model has been recapitulated by sequentially creating cancer-driving mutations in human intestinal organoids [217].

These advances in 3D organoid systems lead us to postulate that integration of the LFS iPSC model with the organoid platform will provide additional opportunities for deciphering the pathogenesis of mutant p53 associated cancers and identifying potential druggable targets (Figure 3). One of the promising combinations will be using 3D cerebral organoids to study LFS-associated brain tumors. PSC-derived cerebral organoids can be grown in a spinning bioreactor system which enables rapid and abundant generation of a 'mini-brain' [218]. Cerebral organoids have been utilized to model neurodevelopmental diseases such as microcephaly [218,219] and lissencephaly [220], and have also been used to identify antiviral compounds against Zika virus [175]. LFS iPSC-derived cerebral organoids hold potential in brain tumor modeling and may clarify the origins of GBM in affected patients.

Recently, researchers have developed **organs-on-chip** systems in an attempt to accurately mimic the cellular environment [221,222]. Organ-on-chip systems integrate cell culture with microfabrication and microfluidics technologies, and allow cells to be cultured in connected chambers. The term organs-on-chip was subsequently used to describe growth of multiple organs on a chip in which various living human cells are cultured in a microenvironment designed to replicate the *in vivo* milieu [221]. Organs-on-chip can represent key functional units of human organs or tissues. With the goal of mimicking the entire human body on a chip, this biomimetic system has great value in drug discovery and testing [223,224]. While many early organs-on-chip systems were developed from primary or transformed cell lines [225–230], newer systems incorporate iPSCs and relevant differentiated tissues into microfluidic devices [231–233]. Mathur *et al*. grew 3D cardiac tissue within a microfluidic device which mimics the blood flow and endothelial barrier. This human iPSC-based cardiac microphysiology system proved particularly valuable in predicting drug-induced cardiotoxicity [231]. In addition, functional differentiation of human pluripotent stem cells directly on microfluidic devices has recently been reported. Through optimal delivery of differentiation medium, Giovanni *et al*. [234] generated functional cardiomyocytes and hepatocytes that showed an expected response to defined drug treatments. We foresee that this powerful technique will provide invaluable information to clarify important missing pieces in the p53/LFS/cancer/development puzzle.

Concluding Remarks

The link between LFS and *TP53* germline mutation has made this hereditary cancer syndrome a unique and useful model in studying p53-associated cancers. Application of patient-derived iPSCs to LFS-associated cancers will be of great value in (i) recapitulating the phenotype of LFS-associated cancer, (ii) elucidating the pathogenesis of mutant p53-associated cancers, (iii) discovering novel mutant p53 gain-of-function, (iv) identifying potential drug targets for mutant

Outstanding Questions

Do cancers derived from LFS iPSC models harbor intratumor heterogeneity resembling the heterogeneity found in patient primary tumors?

Can LFS iPSCs comprehensively recapitulate LFS malignancies in the absence of the microenvironment inside the human body?

Can PSCs engineered to express p53 mutants recapitulate the disease malignancies present in LFS patients and in LFS iPSCs, or is there something else unique about the genetic background of LFS?

Can LFS iPSC-derived tumors offer a practical 'disease-in-a-dish' platform for novel cancer therapeutics discovery?

Can LFS iPSC-derived tumors serve as an alternative PDTX model?

p53, (v) providing unlimited rearrangement-free cell sources for novel drug discovery and compound screening, and (vi) facilitating *in vitro* efficacy and toxicity testing for preclinical compounds. In addition, creating specific *TP53* mutations in WT PSCs using precise genome-editing methodologies will become a valuable resource for developing precision cancer therapy targeting specific p53 gain-of-function mutations.

Variability between individual iPSCs hinders the precise measurement of mutation-associated phenotypes. Generating isogenic control cell lines for disease-specific iPSCs will help to reduce variability caused by genetic background. Precise genome editing enables the creation of isogenic pairs of disease-specific and control iPSCs whose only difference is a disease-causing mutation [235]. The isogenic pairs of iPSCs generated from patients can also be used to test drug toxicity and predict treatment response, and the *in vitro* testing results for each patient will facilitate individual precision treatment.

The fast-growing 3D organoid technology will be increasingly central to cancer models and will assist in identifying and testing potential anticancer drugs. The high structural organization afforded by 3D organoids better mimics organ function than 2D culture, and the complexity of this microenvironment in cancer development cannot be ignored. Recent progress has been made in constructing 3D blood vessels from PSC-derived endothelial and pericytes [236]. The microengineered blood vessels can be lined inside a microfluidic device which can be used to test drug efficacy and study interactions between vascular cells. In the future, vasculature and immune cells may be integrated into a larger tissue construct to advance the development of organs-on-chip systems. Inclusion of the microenvironment into PSC derivatives will offer exciting opportunities to model cancer from a more complex dimension.

In conclusion, LFS disease models offer unique platforms to study and model mutant p53-associated cancers (see Outstanding Questions). Integration of LFS iPSCs and engineered p53 mutant PSCs in cancer modeling offers a valuable source for mechanistic study and drug discovery. We look forward to the wider application of the LFS iPSC model, including 3D organoid and organ-on-chip technologies, in future studies.

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