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Topical Review

Advances and Future Directions for Tuberous Sclerosis Complex Research: Recommendations From the 2015 Strategic Planning Conference

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ABSTRACT

On March 10 to March 12, 2015, the National Institute of Neurological Disorders and Stroke and the Tuberous Sclerosis Alliance sponsored a workshop in Bethesda, Maryland, to assess progress and new opportunities for research in tuberous sclerosis complex with the goal of updating the 2003 Research Plan for Tuberous Sclerosis (http://www.ninds.nih.gov/about_ninds/plans/tscler_research_plan.htm). In addition to the National Institute of Neurological Disorders and Stroke and Tuberous Sclerosis Alliance, participants in the strategic planning effort and workshop included representatives from six other Institutes of the National Institutes of Health, the Department of Defense Tuberous Sclerosis Complex Research Program, and a broad cross-section of basic scientists and clinicians with expertise in tuberous sclerosis complex along with representatives from the pharmaceutical industry. Here we summarize the outcomes from the extensive premeeting deliberations and final workshop recommendations, including (1) progress in the field since publication of the initial 2003 research plan for tuberous sclerosis complex, (2) the key gaps, needs, and challenges that hinder progress in tuberous sclerosis complex research, and (3) a new set of research priorities along with specific recommendations for addressing the major challenges in each priority

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area. The new research plan is organized around both short-term and long-term goals with the expectation that progress toward specific objectives can be achieved within a five to ten year time frame.

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Introduction

Tuberous sclerosis complex (TSC) is a rare genetic disorder (~1:6000 live births) caused by inactivating mutations in either *TSC1* or *TSC2*.[1,2] The proteins encoded by *TSC1* and *TSC2*, hamartin and tuberin, form a complex that negatively regulates the mechanistic target of rapamycin complex 1 (mTORC1).[3] mTORC1 is a kinase that regulates cell growth and anabolic processes in response to nutrient and growth factor stimulation.[3] Clinically, TSC individuals bearing *TSC1* or *TSC2* (*TSC1/2*) mutations develop nonmalignant tumors in multiple organs including the brain, eyes, heart, kidney, skin, and lungs, following a classic tumor suppressor paradigm.[1] However, for many individuals with TSC, the symptoms that most strongly impact quality of life are due to brain involvement, including seizures, intellectual disability, and autism, by mechanisms that are not well understood.[4]

The incidence and severity of TSC manifestations vary widely between individuals, and even between identical twins.[5] This phenotypic heterogeneity is likely due to differences in mutations occurring in *TSC1* versus *TSC2* and other poorly defined factors. TSC is inherited in an autosomal dominant pattern with approximately two thirds of cases arising from *de novo* mutations.[1] In addition, many cases result from genetic mosaicism in which a somatic mutation in *TSC1/2* occurs during early embryonic development.[6,7] In somatic cells, a second-hit event causing complete loss of either *TSC1/2* is typically required to cause unregulated mTORC1 activation and tumor development[1]; heterogeneity arises from stochastic factors that affect the number and distribution of these second hits. Other potential contributors to the heterogeneity include cell-specific responses to the mutation, genetic modifying loci, and developmental and environmental factors, to name a few. This heterogeneity has posed major challenges in identifying effective treatments for TSC.

In 2001, Congress stated its support for the improved detection and treatment of TSC and directed the National Institutes of Health (NIH) to develop a long-range research plan for TSC (S.Con.Res.69, H.Con.Res.25). To assist in developing the first strategic plan for TSC research, the National Institute of Neurological Disorders and Stroke (NINDS), the Tuberous Sclerosis Alliance (TS Alliance), and the NIH Office of Rare Diseases Research convened an international symposium in Chantilly, Virginia, in September 2002 leading to a comprehensive 5- to 10-year research plan for TSC that was published in 2003 (http://www.ninds.nih.gov/about_ninds/plans/tscler_research_plan.htm).

In the Spring of 2014, the NIH, the Department of Defense Tuberous Sclerosis Complex Research Program (DOD TSCRP), and the TS Alliance initiated a new strategic planning effort for TSC that culminated in a workshop on March 10 to 12, 2015, entitled “Unlocking Treatments for TSC: 2015 Strategic Plan” (held in Bethesda, Maryland; Supplementary Data: Appendix 1: Methods; Appendix 2: Workshop Organizing Committee and Working Groups; Appendix 3: Agenda and list of meeting participants). The conference brought together 82 participants including investigators and clinicians with diverse expertise, industry representatives, patient advocates and TSC family members, and representatives from seven NIH Institutes and Centers, the DOD TSCRP, and the TS Alliance. The conference goals included reviewing the state of the TSC research field and progress in reaching the original 2003 research objectives. A major goal was to update the 2003 Research Plan for TSC by identifying critical priorities and new opportunities for the field. Here, we summarize the major workshop outcomes and recommendations to update the TSC Research Plan.

Results

Progress in understanding and treating TSC

The workshop outcomes, described here, included reviewing the state of the TSC field and research progress since publication of the 2003 Research Plan (http://www.ninds.nih.gov/about_ninds/plans/tscler_research_plan.htm).

Elucidation of signaling pathways

Since 2003, tremendous progress has been made in understanding the functions of *TSC1* and *TSC2*, and the molecular and cellular consequences of loss-of-function mutations in these genes. This progress was initiated by seminal findings in *Drosophila* followed by cell culture, and mouse genetic studies indicating that *TSC1* and *TSC2* inhibited cell and tissue growth.[8-11] These studies led to the recognition that TSC1 (also referred to as hamartin), TSC2 (tuberin), and a third protein TBC1D7 form a protein complex (the TSC complex) which acts as a sensor of cellular growth conditions and is an essential negative regulator of mTORC1 (reviewed in the studies[3,12,13]). The TSC complex lies at the heart of a signaling network in which multiple different signaling pathways converge to regulate its function through direct phosphorylation of TSC2. In short, growth-promoting signals from growth factors, hormones, cytokines, nutrients, and cellular energy inhibit the TSC complex, leading to the activation of mTORC1. In contrast, poor growth conditions, such as growth factor or nutrient withdrawal or cellular stress, activate the TSC complex to turn off mTORC1. The TSC complex regulates mTORC1 by acting as a GTPase-activating protein for the Ras-related protein, Rheb, which in its GTP-bound form is an essential activator of mTORC1. Thus, in response to poor growth conditions, the TSC complex, through a GTPase-activating protein domain on TSC2, turns off mTORC1 signaling by stimulating the intrinsic GTPase activity of Rheb, leading to accumulation of GDP-bound Rheb, which cannot activate mTORC1. This regulation appears to occur primarily on the surface of the lysosome, where mTORC1 is independently recruited in response to amino acids.[14] Although our knowledge remains incomplete, the TSC complex is recognized as one of the most highly integrated signaling nodes found in all cells, where its ability to perceive and relay cell intrinsic and extrinsic signals is key to the control of cell, tissue, and organismal homeostasis and growth. We have an even poorer understanding of TSC complex function in the brain; its diverse functions and those of mTORC1 likely underlie the diverse neurological manifestations of TSC.

Clinically, a seminal outcome from this body of work was the recognition that loss of TSC1/2 function causes mTORC1 to become constitutively active in TSC and insensitive to most growth-suppressive signals. This discovery led to preclinical and then clinical trials with allosteric mTOR inhibitors, such as rapamycin (sirolimus) and its analogs (often referred to as rapalogs), for the treatment of TSC manifestations (discussed in the following sections). More recently, novel mechanistic insights in TSC complex function and mTORC1 signaling are fueling new translational directions beyond the rapalogs. For example, novel anabolic functions induced by mTORC1 signaling have been discovered, including *de novo* lipid and nucleotide synthesis, which combined with its established role in induction of protein synthesis, underlie its growth-promoting capacity (e.g., references[15-17]). Disrupting the function of the TSC complex also affects feedback and crosstalk mechanisms within oncogenic signaling networks[18-20] and activates a variety of adaptive response pathways that enable TSC mutant cells to survive the metabolic stress that stems from uncontrolled mTORC1 signaling (e.g., [21-24]). New therapeutic interventions that selectively destroy cells with chronically activated mTORC1 signaling have been suggested by such studies with the hope of eliminating tumors such as renal angiomyolipomas (AMLs) and subependymal giant cell astrocytomas (SEGAs) in TSC patients. Preclinical and clinical studies are underway to test such approaches.

Clinical progress in treating TSC

Remarkable progress in both clinical and translational research has resulted in Food and Drug Administration-approved agents for the treatment of AML, SEGAs, and lymphangioleiomyomatosis (LAM). These efforts have created optimism about the future for additional targeted therapeutic strategies for the tumors that arise in TSC. However, in spite of these advances, there are still key gaps and questions in TSC pathogenesis, and a need to understand better the underlying disease mechanisms, particularly involving the neurological manifestations of TSC, to catalyze development of novel therapeutic approaches.

In the last 4 years, the first three randomized, placebo-controlled, double-blind studies in TSC and LAM were published and have changed clinical practice. For LAM, the Multicenter International Efficacy of Sirolimus (MILES) trial randomized 89 women with sporadic or TSC-associated LAM to receive either sirolimus (rapamycin) or placebo for 1 year, followed by 1 year of observation.[25] Sirolimus stabilized and, by some measures, improved lung function, while lung function in the placebo arm continued to decline.

For SEGAs, the EXIST-1 trial randomized 117 individuals with TSC to either the rapalog everolimus or placebo[26]: 35% of patients in the everolimus group had at least 50% reduction in the volume of SEGAs versus none in the placebo group ($P < 0.0001$). For AML, the EXIST-2 trial randomized 118 individuals with AML to everolimus or placebo[27]: 42% of patients in the everolimus group had at least 50% reduction in the volume of AML versus 0% in the placebo group ($P < 0.0001$).

During this period of clinical progress, novel phenotypes and pathogenic mechanisms of TSC continue to be uncovered. These include the increasing recognition of specific subtypes of renal cell carcinoma in children and adults with TSC,[28,29] the discovery that 80% of women with TSC have evidence of cystic lung disease by age 40 years,[30] and the identification of “second hits” indicating that sun exposure is likely to be a major factor responsible for the development of facial angiofibromas.[31]

Progress in epilepsy associated with TSC

TSC is one of the most common genetic diseases that manifest with epilepsy. Up to 90% of TSC patients develop seizures, most of them starting in infancy. Multiple types of seizures can occur, even within individual patients, and include focal (partial), multifocal, and generalized seizures that may evolve at different ages. Conventional seizure treatments are insufficient in at least one third of patients, causing a significant burden on patients and their families.[32,33] The high prevalence of refractory seizures represents a significant unmet medical need. The mechanism by which TSC causes seizures continues to be uncertain. Tubers and the adjacent (“perituber”) cortex have long been associated with epilepsy. However, epileptiform discharges can occur in areas without tubers, and some TSC patients with epilepsy do not have tubers detectable by magnetic resonance imaging (MRI). For very young children with TSC a common seizure type is infantile spasms. Indeed, any child presenting with infantile spasms should have a thorough evaluation for TSC. Vigabatrin is generally accepted as the first line of medical treatment for infantile spasms in children with TSC although it is not yet clear why this drug is so effective in TSC. The lack of an authentic TSC mouse model with infantile spasms is a major limitation in this area of research.

A previous nonrandomized, open label trial suggested that vigabatrin treatment of TSC infants who developed abnormal electroencephalographs (EEGs) before epilepsy onset could prevent seizure development and improve intellectual outcome.[34] A recent prospective study has identified abnormal EEG as a predictive biomarker of impending clinical seizures in infants with TSC.[35] These studies raise the possibility of seizure prevention in TSC infants if a therapeutic window can be defined and preventive treatment can be given without toxicity. A randomized clinical trial of early intervention with vigabatrin to prevent seizure development in TSC (EPISTOP) is currently ongoing in Europe, and an NINDS—funded trial to prevent epilepsy and improve neurocognitive outcomes in infants with TSC (PREVeNT) is being launched in the United States.

Understanding the neuropsychiatric manifestations of TSC

Significant progress has also been made in understanding the neuropsychiatric manifestations of TSC, with significant impact on how they are managed. Nearly half of TSC individuals are affected with autism spectrum disorder,[36] with symptoms similar to those observed in "idiopathic" autism spectrum disorder.[37] Similarly, intellectual disability is a common problem in TSC. The intelligence/developmental quotient is distributed in a bimodal fashion in TSC, with roughly half of scores fitting a normal distribution with a mean of 92, and half on a distribution with a mean of 42.6.[38] TSC can serve as an effective means to study early stages of autism and intellectual disability because patients can often be diagnosed with TSC in infancy or before birth due to the presence of cardiac rhabdomyomas.[39] There is also high frequency of anxiety, depression, attention deficit hyperactivity disorder and sleep problems in individuals with TSC. This constellation of neurodevelopmental issues in TSC has led to the definition of TSC-associated neuropsychiatric disorders (TAND) as a diagnostic entity. A TAND checklist has come into routine clinical use to assess these issues in TSC and was recently validated.[4,40]

Development of animal models and launch of clinical studies in epilepsy and TAND

Over a dozen different TSC mouse models have been developed that display combinations of epilepsy, hyperactivity, anxiety, learning deficits, repetitive behaviors, and/or social interaction deficits.[41] These models provide insights into the cellular and circuit abnormalities underlying epilepsy and TAND symptoms but have limitations in that they do not entirely replicate the human TSC phenotype (Table 1). Rapalogs are universally effective in preventing or treating seizures and other neurocognitive phenotypes in TSC mouse models. These preclinical studies, and the effectiveness of these medications for AMLs and SEGAs have led to randomized placebo-controlled trials of rapalogs for epilepsy and neurocognition in TSC (NCT01713946, NCT01289912, NCT01730209, NCT01929642). The results of these trials are pending. However, it is becoming clear that the complexity of TSC neurodevelopmental manifestations poses a major challenge for selecting optimal outcome measures in neurocognitive trials. Thus, biomarker studies have been initiated (NCT01780441, NCT01767779) to (1) predict individual patient response to treatment, (2) select subpopulations of patients for clinical trials, and (3) serve as intermediate or surrogate markers of efficacy with the goal of accelerating progress in clinical trials.

Research opportunities and priorities moving forward

The workshop identified five high-priority areas that, if addressed over the next five to ten years, are anticipated to speed progress in our understanding and treatment of TSC. Summarized in the following sections are the key gaps, needs, and challenges recognized to hinder progress in each of these priority areas, along with specific sets of research recommendations for addressing the challenges.

Priority Area I: Understanding phenotypic heterogeneity in TSC

Although a Mendelian disorder, phenotypic heterogeneity is the rule in TSC and manifests as differences in the severity or even presence of symptoms between affected individuals, as well as differences in the severity of different phenotypes within the same individual. For example, one

TABLE 1.
Examples of Preclinical Mouse Models of Tuberous Sclerosis Complex (TSC)

Model	Region/Cell Type	Notable Feature(s)	Phenotypes	Drug Discovery	Reference
Tsc2$^{fl/fl}$; NEX^{Cre}	Forebrain excitatory neurons	Prenatal recombination, astrogliosis	Premature lethality	Everolimus	42
Tsc1$^{fl/fl}$; R26^{tdTom}; Gbx2^{CreER}	Thalamus relay neurons	Temporal mosaicism lineage tracing	Seizures, repetitive grooming	No	43
Tsc1$^{fl/fl}$; CamKIIa^{Cre}	Forebrain excitatory neurons	Postnatal recombination	Kainate seizures	No	44
Tsc1$^{fl/fl}$; L7^{Cre} and *Tsc2$^{fl/-}$; Pcp2-Cre*	Cerebellar Purkinje cells	Purkinje cell degeneration	Impaired sociability repetitive grooming	Rapamycin	45,46
Tsc1$^{f/f}$; Cag-CreERT^{+}	All cells in the adults	Biallelic Tsc1 deletion in the adult	Epilepsy	Rapamycin	47
Tsc2$^{c\text{-}del3}$; Syn-Cre	Neurons	Hypomorphic allele allelic series	Impaired sociability and learning	No	48
Tsc1$^{fl/fl}$; Emx1-Cre	Forebrain progenitors	Megalencephaly disorganized cortex	Seizures, lethality	Rapamycin	49
Tsc1$^{fl/fl}$; Nestin-rtTA; TetOp-Cre	Neurons	Temporal mosaicism	Seizures, hyperactivity	Rapamycin	50
Tsc2$^{fl/-}$; hGFAP-Cre	Radial glial progenitors	Megalencephaly, migration and myelination defects, astrogliosis	Lethality	Prenatal vs postnatal rapamycin	51
pCAG-cre:GFP plasmid into *Tsc1$^{fl/fl}$*	Single-cell deletion	*In utero* electroporation	Heterotopic nodules with cytomegalic neurons	No	52
Tsc1$^{fl/fl}$; GFAP^{Cre} and *Tsc2$^{fl/fl}$; GFAP-Cre*	Astrocytes, neurons	Astrogliosis	Seizures, lethality	Rapamycin	53,54
Tsc1$^{+/-}$ and *Tsc2$^{+/-}$*	All cells	No obvious pathology	Cognitive deficits	Rapamycin	55,56
Tsc1$^{fl/fl}$; Syn-Cre	Neurons	Migration and myelination defects, astrogliosis	Tremor, lethality, seizures	Rapamycin, everolimus pharmacokinetics	57
Tsc1$^{fl/fl}$; GFAP^{Cre}	Astrocytes, neurons	Astrogliosis	Seizures	No	58

individual with TSC may show autistic features without epilepsy or intellectual disability, whereas another may have epilepsy but not autism spectrum disorder. Phenotypic heterogeneity in TSC is thought to result from genetic factors (e.g., type of mutation in *TSC1*/*TSC2*, modifiers, mosaicism), environmental factors such as immune activation or seizures within sensitive periods of brain development, and stochastic factors such as timing and tissue distribution of second-hit events. Understanding phenotypic heterogeneity in TSC is crucial for improving knowledge about underlying mechanisms and natural history and for developing optimal prognostic tools, biomarkers, and targeted treatments for the disorder. Accordingly, the workshop identified two short-term and two long-term goals that would address the mechanisms and implications of this heterogeneity (Table 2).

The first short-term goal is the development of a biobank/database to serve as a repository for biological samples (e.g., DNA, blood and other tissue samples) from individuals with TSC and associated genetic and clinical data for open dissemination among TSC investigators. The TS Alliance has taken a leadership role in the organization of this important resource, which will require continuing development and curation to maximize its impact for studies of phenotypic heterogeneity in TSC. A second and related short-term goal involves leveraging the power of new sequencing technologies (e.g., whole genome or whole-exome sequencing) for deeper genetic analysis of TSC families and expanding the capability of the genetic testing community for routine detection of mosaic mutations and other detailed mutation assessments in TSC. Until recently, most genetic diagnostic laboratories had limited ability to identify mosaicism or rarer TSC mutations, which has hampered our full understanding of the genetic architecture of TSC and associated genotype–phenotype relationships. For example, mosaicism appears to be relatively common in TSC, and it may be associated with a milder phenotype than nonmosaic TSC.[7,59]

Using these important resources (bio/databank and enhanced genetics analysis), the workshop identified two long-term research goals that respectively seek to tackle the genetic and environmental causes of phenotypic heterogeneity in TSC (Table 2). To address these goals, there is a need for comprehensive "omics" and systems-level computational approaches to decipher the complex and intertwined genetic and environmental underpinning of the heterogeneity, particularly by accessing a diversity of clinical samples (e.g., different cell and tissue types) from the biobank. DNA sequencing studies of TSC families, e.g., may identify genetic modifiers that influence the phenotype. In addition, detailed mechanistic studies in animal models are required, ideally conducted in parallel to clinical investigations, to yield insight into the underlying causes of heterogeneity. Such model systems enable in depth exploration of the genetic and environmental causes of heterogeneity and their interactions, in a manner not possible in human studies.

Priority Area II: Gaining a deeper knowledge of TSC signaling pathways and the cellular consequences of TSC deficiency

The TSC complex is a key signaling hub that is modulated through phosphorylation by numerous protein kinases in response to multiple types of extracellular stimuli[12] and that in turn negatively regulates the activity of mTORC1 as described previously. Downstream, mTORC1 regulates a diverse set of cellular functions including protein synthesis, mRNA and ribosome biogenesis, lipid and nucleotide synthesis, mitochondrial metabolism, and autophagy, to name a few.[3,60] Cellular signaling networks are by their nature complicated computational entities, posing challenges for

TABLE 2.
Summary Recommendations: Understanding Phenotypic Heterogeneity in Tuberous Sclerosis Complex (TSC)

Short-term goals:

1. Establish a Bio/Data repository to promote sharing of information/resources and include:
   - a central database for linking clinical/phenotypic information to sequence data and biospecimens
   - genetics/genomics data from TSC probands/families (e.g., DNA/RNA sequences)
   - a rich diversity of patient-derived cell lines, biospecimens, and tissues
2. Expand the use of next-generation sequencing technologies for deeper genetic analysis of TSC families, including routine genetic detection of mosaicism and rarer forms of TSC mutations

Long-term goals:

3. Use computational and "omics" approaches with resources from the Bio/Data repository to investigate the genetic causes for the heterogeneity between and within individuals including the role of:
   - specific *TSC1/2* patient mutations on the phenotype
   - mosaicism
   - genetic modifiers/secondary loci that contribute to the severity of the phenotype
   - epigenetics
4. Explore nongenetic contributions to phenotypic heterogeneity in TSC including the role of:
   - environmental exposures, inflammation/infection, tumor microenvironment, endocrine and stress responses, sleep, dietary influences, etc.
   - epilepsy on neurocognitive development
   - development (age of patient)

unraveling their functions. By mechanisms that are poorly understood, the activities of diverse upstream regulators and downstream effectors of the TSC complex are influenced by the many genetic and environmental sources of heterogeneity in TSC (Priority Area I), which collectively give rise to heterogeneity at the cellular, circuit, and network levels and consequently in the clinical manifestations of TSC. The workshop identified both short-term and long-term goals that would help basic scientists and clinicians to gain a deeper understanding of altered signaling pathways in TSC and their clinical consequences (Table 3).

Of immediate benefit would be a better toolbox for TSC researchers including antibodies, constructs, pharmacological grade compounds, and novel reporters that, in conjunction with the resources from the bio/databank (Priority Area I), could be used to monitor and probe signaling pathways and cellular functions that are known to be regulated by the TSC complex and mTORC1. These tools should be openly disseminated in the form of an easily searchable database to enable easy access.

The workshop identified multiple long-term research goals that are imperative for unraveling the extraordinarily complex and dynamic nature of the TSC signaling network. These objectives include obtaining detailed structural knowledge of the large (~2 MDa) TSC protein complex[14] and quantitatively assessing the TSC signaling network using proteomics, phosphoproteomics, metabolomics, transcriptomics, and translatomics in combination with systems/computational analytic approaches. It will also be important to identify the key upstream signaling inputs and to decipher the role of mTORC1-independent pathways in TSC. Harnessing the computational power of bioinformatics approaches will be critical to these endeavors, as well as studying a diversity of cell types and in both heterozygote and homozygote mutant TSC cells as highlighted below.

It is becoming increasingly clear that different cell types can exhibit different phenotypes in response to *TSC1/2* deficiency and mTORC1 activation. For example, in response to *TSC1/2* loss, basic cellular processes, such as autophagy, are differentially perturbed in neuronal versus non-neuronal cells.[22,61] Different neuronal cell types (e.g., hippocampal versus cerebellar Purkinje neurons) can also respond very differently to *TSC1/2* loss, e.g., regarding perturbations in dendritic spine dynamics.[45,57] Moreover, in contrast to tumor formation in TSC, which requires second-hit events (discussed previously), a number of studies have documented the deleterious effects of single copy loss of *TSC1/2* (haploinsufficiency) on synaptic connectivity and behavior in TSC mouse models.[55,56,62,63] Further analysis and mechanistic understanding of this phenomenon is required and may help to explain multiple aspects of TAND. These cell type and regional differences in responding to TSC mutations highlight the importance of investigating the impact of mutations in different spatial and temporal settings, in diverse cell types and at specific stages of development.

TABLE 3.
Summary Recommendations: Gaining a Deeper Knowledge of Tuberous Sclerosis Complex (TSC) Signaling Pathways and the Cellular Consequences of TSC Deficiency

Short-term goal:

1. Develop a better toolbox for TSC researchers
    - in addition to a clinical Bio/Data repository, establish a repository and database of available molecular tools/reagents (e.g., antibodies, tool compounds, reporters, constructs), cell lines and animal models to promote sharing and dissemination of information about these resources

Long-term goals:

2. Delineate TSC-dependent signaling networks quantitatively in both homozygous and heterozygous disease-relevant cells
    - determine the 3-dimensional structure of the TSC protein complex and define the molecular basis of its interactions with Rheb and other proteins
    - employ unbiased “omics” (e.g., proteomics, phosphoproteomics, metabolomics, transcriptomics, translatomics) and systems/computational approaches to understand the cellular consequences of mutations in *TSC1* and *TSC2*
    - tease apart the role of mechanistic target of rapamycin complex 1 (mTORC1)—dependent and mTORC1-independent pathways, and the role of mTORC2 in TSC
    - understand the upstream regulators of the TSC complex in different contexts

3. Develop a thorough understanding of cell- and tissue-specific manifestations of TSC deficiency; e.g., delineate:
    - cell-specific differences in the consequences of *TSC1/2* mutations; e.g., phenotypic differences in neuronal versus non-neuronal cells, in excitatory versus inhibitory neurons, and so forth
    - the role of homeostatic or compensatory/aggravating mechanisms (including interactions with other pathways) in modifying the impact of mutations within cells
    - developmental influences on the phenotype

4. Understand non-cell autonomous effects of *TSC1/2* deficiency; e.g., understand:
    - how *TSC1/2* deficient cells impact the functioning of neighboring cells (e.g., wild-type cells in mosaicism) or modify circuit/network dynamics in the brain
    - the role of the microenvironment in LAM and TSC pathology: e.g., interactions with the tumor stroma and inflammatory cells; lung destruction and lymphangiogenesis in LAM; and angiogenesis in AML and skin lesions
    - the role of neuron—glial interactions in the TSC phenotype

A major gap, however, is the limited availability or difficulty in deriving cultures from some cells or tumors (e.g., TSC-associated SEGAs, AMLs, angiofibromas, LAM).[64,65]

Another long-term goal is to identify non-cell autonomous effects of *TSC1/2* deficiency (both heterozygous and homozygous) in available cell models, animal models, and patient-derived cells and tissues (Table 3); i.e., how does dysregulation of the TSC signaling network in one type of cell impact the function of other cells in a tissue?

Priority Area III: Improving TSC disease models

The workshop participants identified the need for both new cellular and animal models of TSC (Table 4). A technology that may prove transformative for TSC research is the use of induced pluripotent stem cells.[66] This approach is based on the ability to reprogram somatic cells (e.g., skin fibroblasts or lymphocytes) obtained from patients with diseases such as TSC into stem cells. The technology for the generation of these lines is now fairly robust, but their utility and reproducibility in the analysis of human phenotypes is still under investigation. Importantly, the use of genomic engineering technologies such as TALEN (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats) enables the generation of paired isogenic control and TSC lines that harbor specific mutations, enhancing utility. The future availability and distribution of induced pluripotent stem cell lines from TSC patients curated with associated phenotype/genotype data and the validation of findings using multiple TSC patient cell lines will add a crucial dimension to boost translational research in TSC.

Numerous (more than 20) distinct TSC animal models (primarily mouse) have been generated since 2002, which capture various features of the human disease (Table 1 includes 14 models with neurological phenotypes). When interpreted within the scope of their limitations, these models provide valuable insight into underlying disease mechanisms. The current models use a variety of genetic technologies, including conditional alleles that allow for cell type–specific or regional deletion of *Tsc1/2* and the concomitant dysregulation of the mTOR pathway or permutations that capture the genetic mosaic nature of TSC.[59] Not surprisingly, given the genetic and phenotypic heterogeneity of the human disorder (not to mention the influence of evolution particularly on brain development), no single genetic model recapitulates precisely the full pathology seen in human TSC; collectively, however, the models can provide important insights into TSC disease biology. Two phenotypes that converge in nearly all the brain models (Table 1) are increased levels of phospho-S6 and increased cell growth, the molecular and cellular consequences of uncontrolled mTORC1 signaling. Most models also have an

TABLE 4.
Summary Recommendations: Improving Tuberous Sclerosis Complex (TSC) Disease Models

Short-term goals:

1. Use rigorous study design and transparent reporting to advance the most robust and reproducible preclinical concepts to clinical testing; e.g., for preclinical therapeutics development:
   - ensure blinding, randomization, appropriate controls, power, and statistics
   - use human-relevant doses in animal models and incorporate pharmacokinetic (PK)/pharmacodynamics (PD) measures
   - consider both timing (in relation to symptom onset or treatment windows) and duration of treatment, corresponding as closely as possible to the clinical indication
   - identify robust and reproducible phenotypes (e.g., conserved across multiple TSC mouse models and/or background strains, or across species) to increase confidence that preclinical results will translate to humans
   - align clinical and preclinical studies, adopting “reverse translation” strategies when possible; e.g., clinical biomarkers or intermediate phenotypes identified from TSC patients (discussed below) that can be recapitulated in animal models
   - replicate promising preclinical treatment findings in more than one model and in independent laboratories
2. Establish a “Preclinical Trials Network” to accelerate translation to human studies
   - include expertise in different organ systems
   - include collaboration with the TSC Clinical Research Consortium

Long-term goals:

3. Develop new animal models that represent the specific clinical features of TSC (e.g., subependymal giant cell astrocytoma [SEGA], angiomyolipoma [AML], lymphangioleiomyomatosis [LAM], cardiac rhabdomyomas, cortical tubers, infantile spasms, TSC-associated neuropsychiatric disorder [TAND]) and can better inform clinical translation
   - develop models with improved construct validity (e.g., mosaic models, patient-specific mutations)
   - in addition to mouse models, diversify the “animal model toolbox” by developing rat and nonrodent mammalian models for preclinical studies
   - employ zebrafish/*Drosophila* models to facilitate the study of genetic modifiers in TSC
4. Develop a diverse set of cell-based models representing different cells, tissues, and organs affected by TSC
   - consider cell of origin
   - human induced pluripotent stem cell (iPSC) lines with paired single/double hits and isogenic controls
   - patient-derived xenografts (PDX)
   - 3D organ culture and tissue-chip technologies

epilepsy or seizure phenotype (induced or spontaneous), whether targeting gene deletion to astrocytes, neurons, or progenitor cells. Posing a challenge for studies of TAND, a more limited subset has aberrant behavioral features. Multiple non-brain TSC models have also been developed and used successfully for therapeutic testing (e.g., rapamycin for tumor elimination). However, there are no practical models yet that replicate human AML or LAM, highlighting the need to develop better tumor models of TSC. Hence, the workshop participants recognized the need to develop and disseminate a diverse "toolbox" of models to accelerate translational progress in TSC.

Given the many failures to translate findings from animal models to humans,[67-69] mouse model development is currently in a stage of re-examination and revitalization. For example, the field is recognizing the need to identify robust and reproducible phenotypes, particularly those that are conserved across multiple mouse models and strains or even across species, to increase confidence that preclinical results will translate to humans.[70,71] In that light, many drug development programs are moving away from using complex, highly strain-dependent behaviors in rodent efficacy assays (e.g., reversal of social impairments in mice), relying instead on more robust, evolutionarily conserved phenotypes that capture underlying biology or circuit function.[72] Reverse translational and iterative approaches, e.g., identifying clinical biomarkers or intermediate phenotypes in TSC patients (Priority Area IV) that can be recapitulated in animal models, are also being explored to improve the informative value of both preclinical and clinical markers used in translational research.

Furthermore, preclinical studies are often not rigorously designed or reproducible. Consequently, the NIH and leading scientific journals recognize the urgent need to submit preclinical studies to the same standards of rigor (e.g., blinding, randomization) and transparency that are expected of human clinical trials[73,74]; also, see recent NIH guidelines: http://grants.nih.gov/reproducibility/index.htm. In addition, when testing the efficacy of pharmacologic interventions in preclinical models, it is imperative to obtain pharmacokinetic (PK) end points in plasma and/or the tissue in which the drug target is expressed[75-77] and directly compare PK with pharmacodynamics (PD) end point(s). Finally, the most promising treatments should undergo replication, ideally in an independent laboratory and/or using another TSC model, before the advancement to late-stage translational or clinical testing. The lack of PK/PD relationships, the absence of appropriate controls, and the lack of randomized, blinded, and sufficiently powered preclinical studies are likely to undermine translational success in TSC.

There is recognition of the financial and logistical challenges for academic laboratories to conduct PK/PD and replication studies, along with a need for additional resources and partnerships. Funding agencies including the NIH, DOD TSCRP, and TS Alliance have a number of funding mechanisms that are specifically designed to support such studies (Supplemental Data: Appendix 4). Furthermore, a strong recommendation is to establish a preclinical TSC trials network that has an integrated mouse models consortium component. The network would serve as a centralized resource for existing and new genetic models and provide an in-depth description of the study design, methods, pharmacologic or other agents used, results (including PK/PD and independent replication outcomes), and utility of the models for different preclinical applications. The TS Alliance has taken a leadership role and begun to organize a TSC Preclinical Trials Network to include investigators with expertise in TSC mouse models and the different organ systems affected by TSC along with industry experts to guide drug-discovery criteria. Table 4 lists specific strategies to achieve each of the goals in Priority Area III.

Priority Area IV: Developing clinical biomarkers for TSC

Biomarkers, defined broadly as characteristics of the body that can be measured in relationship to disease, can facilitate advances in a myriad of aspects of clinical care and trials. Biomarkers can be powerful tools in a variety of domains to (1) aid in disease screening and diagnosis (diagnostic biomarker), (2) provide prognostic information about the natural history of disease (prognostic), (3) predict individual treatment response and patient stratification for clinical trials (predictive), (4) yield insights into disease pathogenesis (pathogenic), and (5) serve as predictors of target engagement, PD measures, or efficacy for clinical trials (PD/response). Advances in biomarker development in TSC will provide synergy to all priority areas in TSC (Table 5 outlines specific strategies to achieve this goal).

There are numerous types of biomarkers currently used in TSC clinical practice. For example, imaging modalities (MRI, computed tomography or ultrasound) provide organ-specific measures of tumor burden. Pulmonary function tests are used to measure the severity of LAM or disease progression. Serum vascular endothelial growth factor D, a lymphangiogenic growth factor, facilitates LAM diagnosis and has a potential role in prognosis estimation and prediction of response to sirolimus. Biomarkers are particularly crucial for measuring neurological and psychiatric manifestations of TSC. The EEG serves as an index of the activity of large populations of neurons acting in synchrony and is an important measure of seizure activity in TSC. In addition, EEG signals, commonly quantified as event-related potentials or by spectral analyses can provide a window for detecting cortical circuitry defects or abnormal functional connections in the human brain. Human EEG measures have been recapitulated in TSC mouse models[53,78,79] potentially serving as important tools for reverse translational studies. Functional and structural MRI can also serve as biomarkers to assess connectivity in the human brain. Prospective biomarker studies are ongoing in TSC using MRI and EEG (NCT01767779, NCT01780441).

However, there remains a clear unmet need for improvement of existing biomarkers and for development of novel clinical biomarkers in multiple aspects of TSC. The field lacks sufficient biomarkers of disease burden and activity, including dynamic measures of disease state (e.g., beyond static imaging of tumors). For example, a current limitation is our ability to assess lung involvement and disease progression by LAM in TSC. New tools are also required to better assess the clinical response to rapalogs and other targeted therapeutics, including biomarkers to measure target engagement, PD response, and to provide precision in assessing the clinical response to treatment. Improved measures of neural circuit function and functional connectivity, e.g., would have broad utility for

TABLE 5.
Summary Recommendations: Developing Clinical Biomarkers for Tuberous Sclerosis Complex (TSC)

Short-term goals:

1. Facilitate biomarker discovery projects by supporting:
    - longitudinal collection of biospecimens for aforementioned Bio/Data repository
    - the genotyping of patients or collection of samples in ancillary clinical studies
    - unbiased screening of biospecimen samples and tissues
    - additional data mining efforts in ongoing clinical studies

2. Convene a TSC biomarkers workshop
    - take advantage of biomarker expertise in related neurodevelopmental or cancer/mechanistic target of rapamycin (mTOR) disorders

Long-term goals:

3. Develop strategies to assess signaling biology, target engagement, physiology, pathology across the domains of TSC, and in accessible tissue compartments; examples include research aimed to:
    - develop more sensitive (e.g., novel positron emission tomography radiotracers) and/or non-invasive, accessible measures (e.g., skin imaging, electroencephalograph [EEG]) of TSC pathology including tools for early detection and screening
    - develop dynamic measures for functional assessment of TSC manifestations (beyond anatomic measures of pathology) and response to interventions
    - develop proximate readouts of target engagement and pharmacodynamics
    - better understand the pharmacodynamics of rapalogs
    - define excellent versus poor responders, extremes of phenotypes, and other aspects of clinical heterogeneity for patient stratification in clinical trials
    - develop measures that can be implemented across multiple sites and repeated over the life span

4. For neurological manifestations of TSC, develop and validate biological, molecular, neurophysiological (e.g., EEG), and imaging markers in conjunction with behavioral outcomes
    - develop “next-generation” imaging tools to measure circuit function in the TSC patient population; e.g., motion insensitive, faster scans
    - conduct longitudinal studies to assess neurodevelopmental trajectories

diagnosis, prognosis, and prediction in TAND and for use as PD biomarkers in clinical trials.

Given the clinical heterogeneity in individuals with TSC, the development of risk stratification tools as predictive biomarkers of prognosis and clinical phenotype remains a high priority and one that will be required for prevention trials. Examples include the early characterization of slowly versus rapidly progressing tumors and markers that accurately predict those at high risk of developing epilepsy, autism spectrum disorder or other features of TAND, renal cell carcinoma, and clinically significant LAM. Improved ability to differentiate and predict excellent and poor responders to rapalogs would aid in patient selection for trials, help stratify TSC patients for personalized dosing, and ultimately facilitate more efficient trial design.

The workshop participants identified a number of strategies to facilitate biomarker discovery and development in TSC (Table 5), recognizing that such advances would provide synergy to other priority areas in TSC. There is also a need to develop translational biomarkers for preclinical models and human studies, incorporate biospecimen collection in clinical trials, and promote the translation of biomarkers into clinical practice. Appendix 4 (Supplementary Data) lists some of the current funding programs that potentially could support biomarker development in TSC.

Priority Area V: Facilitating therapeutics and clinical trials research

Despite tremendous progress in treating the tumors in TSC, a number of crucial gaps remain especially for treating the neuropsychiatric manifestations of TSC. For example, there is a need to develop more sensitive and reliable end points in clinical trials of TAND, and to incorporate biomarkers in the design of clinical trials. In addition, the identification of novel therapeutics beyond the rapalogs would potentially benefit all manifestations of TSC. To facilitate TSC therapeutics and clinical trials research, several short-term and long-term recommendations were developed (Table 6). Since 2011, there is an ongoing TSC Clinical Research Consortium funded by the NIH focusing on epilepsy and neuropsychiatric aspects of TSC. This Clinical Research Consortium has launched several studies in epilepsy and TAND in collaboration with the TS Alliance. Recommendations that can be adapted in the short term include significantly broadening the already existing Clinical Research Consortium in terms of the number of participating sites and areas of research. The efforts of the Clinical Research Consortium can be used to expand clinical research into non-neurological manifestations of TSC (Table 6). To guide these expanded efforts, the steering committee should be broadened to include consultants with links to preclinical pharmaceutical and biotech companies. These consultants will provide valuable input such as drug development pipeline information and patient-perceived needs. In future clinical research, efforts should be made to recognize and include broader aspects of TSC and to gather more exploratory disease end points.

Longer term recommendations include development of methods to capture the effects of clinical interventions, including therapeutic and behavioral interventions. The

TABLE 6.
Summary Recommendations: Facilitating Therapeutics and Clinical Trials Research

Short-term goals:

1. Broaden the TSC Clinical Research Consortium to include:
    - experienced clinical trialists and members of the TS Alliance Professional Advisory Board
    - preclinical investigators and industry representation
2. Recognize all aspects of TSC disease manifestations in clinical trials
    - for example, Tuberous Sclerosis Complex (TSC)-associated neuropsychiatric disorder (TAND) checklist should be included in interventional studies
    - assess tumors and other organ involvement in neuropsychiatric trials

Long-term goals:

3. Before launching pivotal trials, conduct exploratory clinical studies to understand and determine optimal:
    - dosing, timing, and duration of intervention (in conjunction with pharmacokinetic [PK]/pharmacodynamics [PD] measures) for a given manifestation of TSC
    - patient population; e.g., age, mutation type, stratifying excellent versus poor responders
    - biomarkers and clinical end points for trials
4. In addition to treatment trials, there is an urgent need to develop:
    - biomarkers and surrogate markers that target most of the patients and are validated by PD response and treatment outcome
    - more sensitive behavioral and cognitive outcome measures for clinical trials in TAND
    - combination therapies; e.g., drug therapy combined with behavioral/cognitive interventions for TAND
    - preventative therapies; e.g., determine whether early treatment can prevent progression to later stages in TSC and lymphangioleiomyomatosis (LAM); prevention of epilepsy in TSC
5. Follow and/or optimize the outcomes of existing clinical interventions over the long term; e.g.,
    - conduct further studies to optimize the use of rapalogs and other mechanistic target of rapamycin (mTOR) inhibitors in LAM and TSC; e.g., determine the lowest effective dose; safety and efficacy with long-term use

TAND checklist is an example of a successful measure in this regard. An organized but easy to implement approach can accelerate improvements in patient care and be used to facilitate longer term effects that extend beyond financial barriers of funding cycles. Surrogate end points including biomarkers and efforts aimed at disease prevention are critical to daily clinical care and research. Finally, long-term prevention trials will need a method for funding to find preventative therapies (Supplementary Data: Appendix 4).

Conclusions

The workshop summary reported here describe a research strategy aimed at addressing the numerous medical and neuropsychologic burdens associated with TSC while deciphering the biology underlying phenotypic heterogeneity. It is important to restate the major advances in TSC therapeutics that have occurred in the past ten years, including use of rapalogs for multiple aspects of TSC and use of vigabatrin for treatment of TSC infantile spasms. In spite of these advances, the TSC disease burden remains large. However, when the causes of interindividual variability are understood, individualized prognoses, surveillance, and treatments can be developed based on biomarkers that measure one's risk for each of the various manifestations. As new ways of treating each manifestation are developed through research on the different aspects of TSC, treatments can be personalized to maximize the risk—benefit ratio for each individual. We are not there yet—but here we propose a research strategy designed to improve our understanding and treatment of TSC.

An important outcome of the workshop was the identification of key gaps and needs that cross all aspects of the disease, including better systems to acquire, annotate, and distribute biospecimens, improvement in animal models, development of better systems for standardized preclinical studies, and a broader clinical trials network including non-neurological manifestations of TSC. Focused workshops addressing a biospecimen repository and a preclinical trial consortium were held in October 2015.

To turn these research goals into accomplishments will require coordinated efforts of basic scientists, clinical researchers, academic centers, and industry partners. By reducing the barriers between institutions and disciplines, enhancing communication and collaboration, and promoting multi-site preclinical and clinical trials, the TSC research community is likely to build on the tremendous progress that has been achieved since the 2002 workshop. Such a collective effort is required to improve the lives of individuals and families affected with TSC.

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Supplementary data

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