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INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant multisystem disorder caused by loss of either TSC1 or TSC2 function (Tsai and Sahin, 2011). TSC affects 1/6,000 individuals worldwide and affects multiple organs including the brain, skin, eyes, kidneys, heart, and lungs (Crino et al., 2006). TSC patients present with epilepsy (~90%), intellectual disability and autism (~50%), and other disorders including sleep disruption, attention-deficit hyperactivity disorder, and anxiety (Han and Sahin, 2011). The neuropathological findings in TSC are cortical tubers, subependymal nodules and subependymal giant cell astrocytomas (SEGAs) (DiMario, 2004). Another important yet not well-studied feature of TSC pathology in brain is hypomyelination (Ridler et al., 2001). Most recently using diffusion tensor imaging we observed abnormal white matter microstructure in patients with TSC that have autism compared to TSC patients without autism (Lewis et al., 2013; Peters et al., 2013). To uncover the underlying molecular mechanisms of hypomyelination in TSC, we investigated the role of neuronal factors affecting oligodendrocyte development in our *Tsc1^{cc}Syn1Cre⁺* mouse model, which lacks Tsc1 expression only in neurons. Here we show that, neurons lacking Tsc1 secrete excessive amounts of connective tissue growth factor (CTGF), which in turn blocks the maturation of oligodendrocytes, and thus myelination both *in vitro* and *in vivo*.

KEYWORDS

Tuberous Sclerosis Complex, myelination, CTGF

ACCOMPLISHMENTS:

We will review the accomplishments to date in each aim, as taken from the SOW (in **bold**). The figures are in the appendices.

Aim 1A: To determine the role of CTGF in hypomyelination in the mouse model.

I. We will generate two independent CTGF-shRNAs and confirm their efficacy in vitro.

We had previously shown that supernatant from HEK293T cells that express full length CTGF or commercially available recombinant CTGF reduce the number of MBP+ oligodendrocytes in cultures (Figure 1a-d). In addition, we had showed that adding neutralizing anti-CTGF antibody to conditioned medium from TSC-deficient neurons was able to ameliorate the effect of this conditioned medium on oligodendrocyte maturation and MBP expression (Figure 1e-f). We had proposed knocking down CTGF in TSC-deficient neurons as one way to verifying the role of CTGF in vitro. We did not proceed with these in vitro experiments since the *in vivo* experiments detailed below gave extremely compelling results allowing us to focus on the role of CTGF in the mouse model.

II. We will generate AAV2-CTGF-shRNA to reduce the expression of CTGF in vivo. AAV2-expressing scrambled shRNA as a control to the contralateral hemisphere. Alternatively, we will generate mice missing both Tsc1 and Ctgf genes under the Syn-Cre promoter (see grant proposal for details). Then we will follow look at MBP staining in the double knockout mice compared to single Tsc1 knockout mice.

We crossed *Tsc1;Syn1Cre⁺* mice with *CTGF^{ff}* mice (Kapoor et al., 2008) to generate the following groups of mice: wild type for both genes (*Tsc1^{ww};Syn1Cre⁺;CTGF^{+/+}*), mutant for *Tsc1* and wild type for *CTGF* (*Tsc1^{cc};Syn1Cre⁺;CTGF^{+/+}*) and mutant for both genes (*Tsc1^{cc};Syn1Cre⁺;CTGF^{ff}*). To our knowledge, this is the first mouse model lacking CTGF only in neurons. We stained the brain sections of control, mutant and double mutant with the MBP antibody to visualize myelination and found that loss of CTGF partially rescued the hypomyelination phenotype as assessed by the increased MBP

signal (Figure 1g-h). Interestingly, the number of mature oligodendrocytes, which were counted by CC1 staining, did not change between the mutant and double mutant (Figure 2a-c). We used CC1 expression to detect the mature oligodendrocytes by immunohistochemistry. This suggests that CTGF blocks the differentiation of oligodendrocytes to myelinating oligodendrocytes; however, another yet unknown factor affects the development of oligodendrocytes in mice lacking *Tsc1* in neurons. Moreover, the loss of CTGF in neurons, which are wild type for *Tsc1*, also enhanced myelination (Figure 2d-f). Together these data suggest that the neuronal CTGF is necessary for blocking myelination *in vivo*.

These results beg the question how TSC-deficiency regulates CTGF expression. We focused on the role of SRF. Previous reports show that the SRF functions as the repressor of *Ctgf* transcription (Stritt et al., 2009). Importantly, a previous study showed that neurons lacking SRF have higher expression of CTGF and thus blocks the maturation of oligodendrocytes (Stritt et al., 2009). We therefore analyzed the levels of SRF both in our mutant mice and in primary cortical neurons lacking *Tsc2*. Both SRF protein and transcript levels were decreased in *Tsc2* KD neurons compared to the control neurons (Figure 3a-c). In addition, we checked the transcript levels of other targets of SRF, *Egr1* and *Cyr61*. SRF functions as a transcription activator of *Egr1* (Kim et al., 2008), whereas it suppresses the transcription of *Cyr61* (Stritt et al., 2009), which is in the same CCN family of proteins as CTGF (Jun and Lau, 2011). In *Tsc2* KD cortical neurons, both the transcript levels of SRF and *Egr1* are decreased, whereas *Cyr61* is increased as *Ctgf* (Figure 3c). Moreover, when stained with SRF antibody, compared to the control mice, the mutant mice brain sections showed decreased intensity, suggesting SRF expression is diminished both *in vivo* and *in vitro*. Together, our data suggest that the upregulation of CTGF in *Tsc*-deficient neurons may be due to the downregulation of its repressor, SRF.

Aim 1B: To test whether CTGF expression is altered in human TSC brain.

We have initiated staining of CTGF in tuber specimens from TSC patients taken at the time of epilepsy surgery. We have so far stained paraffin sections of tubers from TSC patients and detected that CTGF is expressed in the tuber. We found that all phosphor-S6 positive cells are also CTGF-positive (Figure 4).

Aim 2: To examine the mechanisms by which CTGF regulates oligodendrocyte differentiation.

Our preliminary data from our first-year report suggests that the Mod-IV, which is one of the domains of CTGF is responsible for inhibiting oligodendrocyte maturation. To address this question systematically, we have been generating CTGF expression constructs, which express different domains of CTGF (Mod-I, Mod-II, Mod-III, Mod-IV and their combinations) to test the mutual and/or complementary effects of these different domains on oligodendrocyte maturation. So far we have generated the following FLAG-tagged constructs: Full-length, Module I, Module I+II. The cloning for the rest of the constructs is in progress. We had showed that the CM collected from HEK293T cells expressing the full-length FLAG-CTGF affects the maturation of oligodendrocytes. We will now test the individual modules for their effect on oligodendrocyte maturation assay.

IMPACT

In this study, we report that the double mutant, which lacks both *Tsc1* and CTGF in neurons rescue myelination. Moreover, we continue to work on the upstream regulators of CTGF expression and we focused on SRF pathway. In addition to our preliminary findings on SRF pathway in our previous report, here we show that SRF levels are decreased *in vivo* in mutant mice, and this can be rescued by rapamycin treatment. The crosstalk between SRF and mTOR pathways will not only provide a better understanding of regulation of CTGF expression but also help us to understand the basic control mechanisms of cell growth and survival. Better understanding of the role of CTGF in

myelination may provide insights and new therapeutic options for TSC and other diseases affecting the white matter.

CHANGES/PROBLEMS:

None

PRODUCTS:

We submitted the manuscript describing our findings for publication.

We have also published two review papers related to this topic and acknowledged the funding from the DOD:

[Autism and the synapse: emerging mechanisms and mechanism-based therapies.](#)

Ebrahimi-Fakhari D, **Sahin M.**

Curr Opin Neurol. 2015 Feb 19. [Epub ahead of print]

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Davis PE, Peters JM, Krueger D and **Sahin M.** Tuberous Sclerosis: A new frontier in targeted treatment of autism. Neurotherapeutics (in press).

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

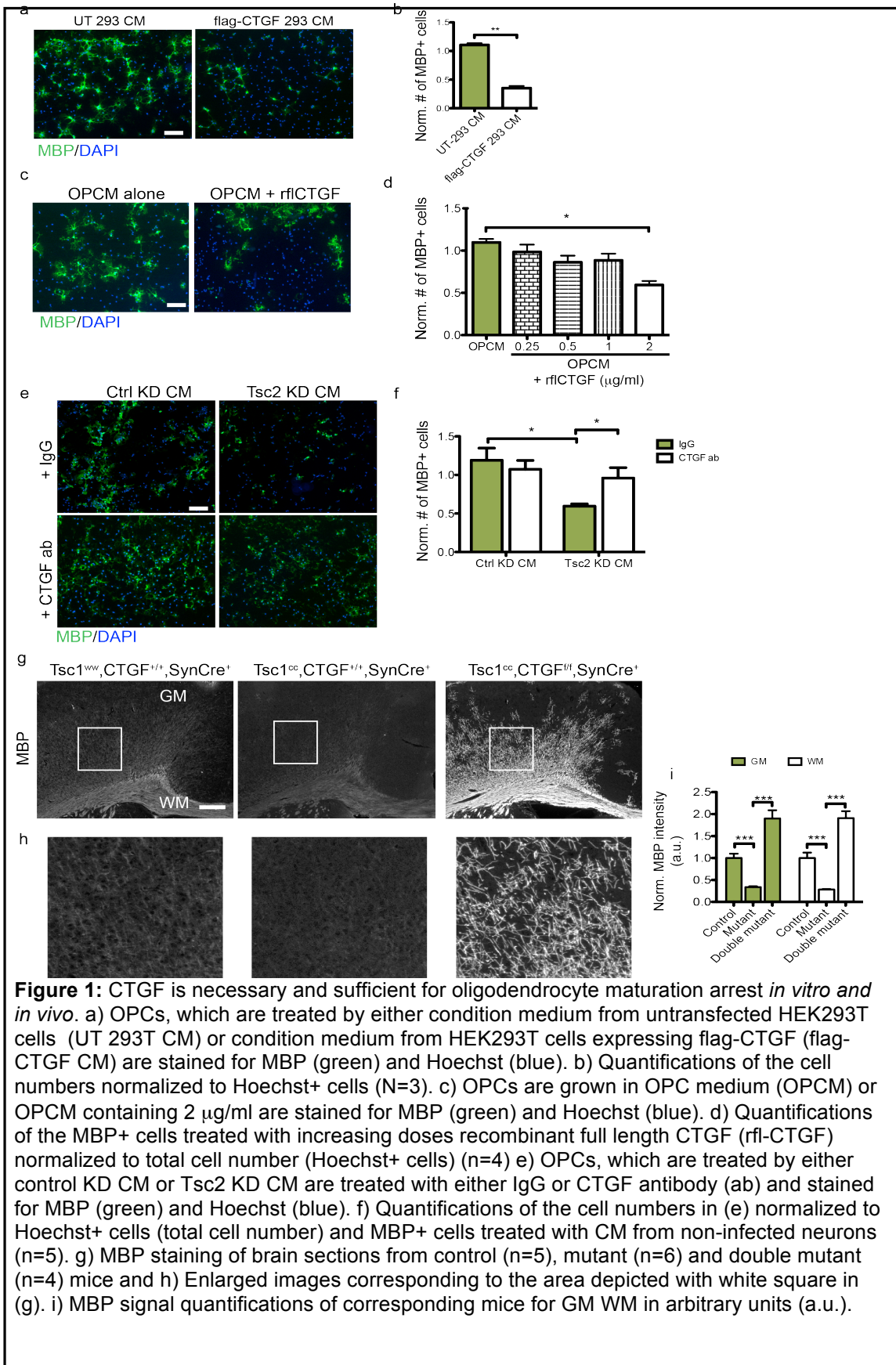
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SPECIAL REPORTING REQUIREMENTS

None

APPENDICES:

Figures 1-4



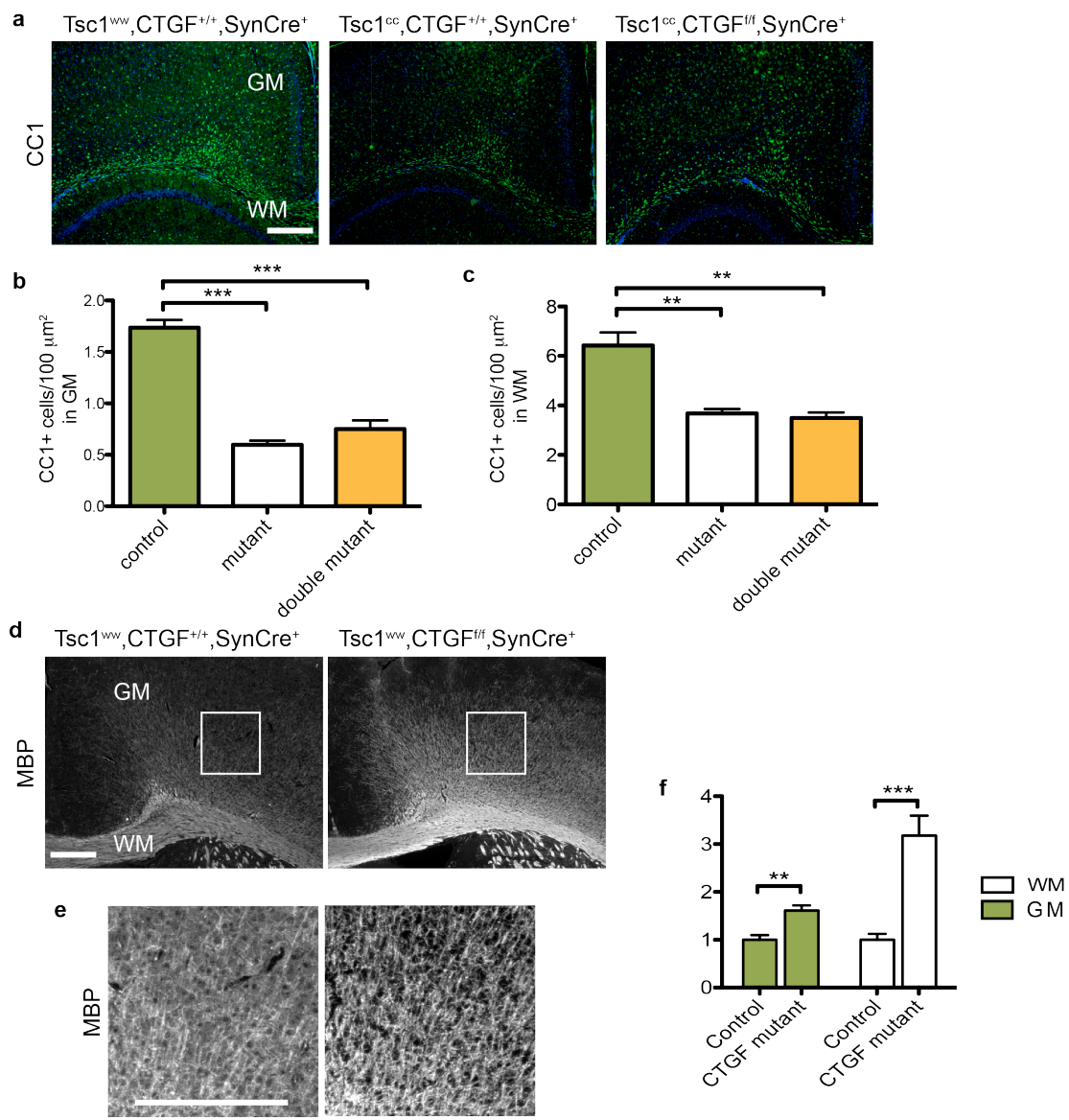
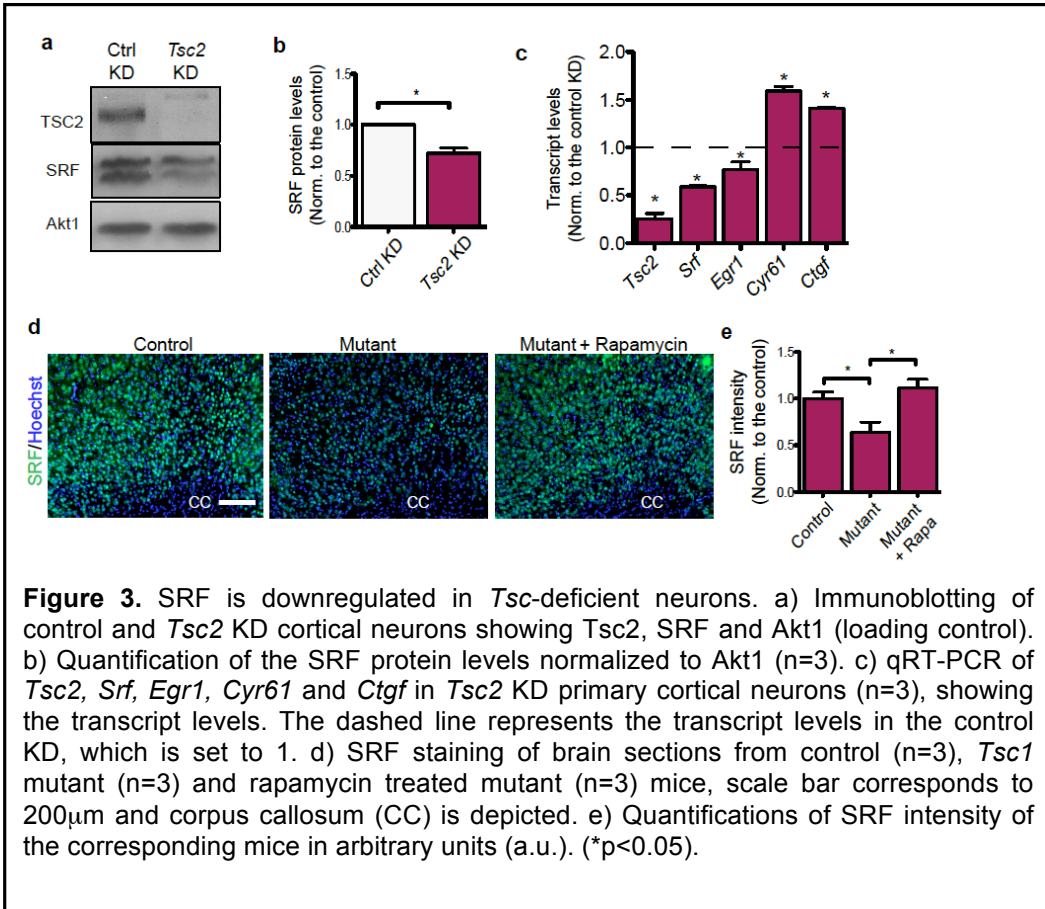
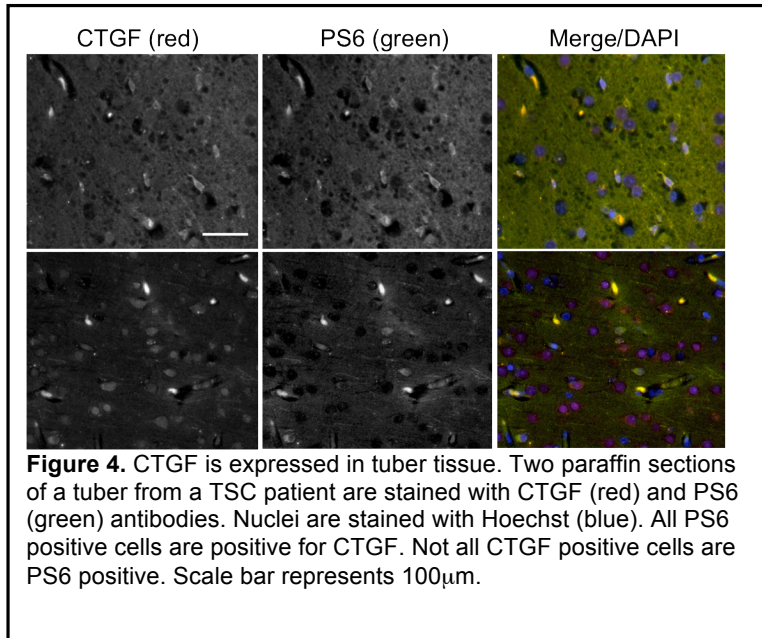


Figure 2. The number of CC1 positive mature oligodendrocyte number decreases in the *Tsc1* mutant mice, the double mutant, which lacks both *Tsc1* and *CTGF* does not rescue the number of CC1 positive cells (a-c). Mice, which are wild type for *Tsc1* but lacking *CTGF* in neurons, represent higher myelination as MBP intensity increases in the *CTGF* mutant compared to the wild type control (d-f).





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