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# Metformin Acts on Two Different Molecular Pathways to Enhance Adult Neural Precursor Proliferation/Self-Renewal and Differentiation

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## SUMMARY

The recruitment of endogenous adult neural stem cells for brain repair is a promising regenerative therapeutic strategy. This strategy involves stimulation of multiple stages of adult neural stem cell development, including proliferation, self-renewal, and differentiation. Currently, there is a lack of a single therapeutic approach that can act on these multiple stages of adult neural stem cell development to enhance neural regeneration. Here we show that metformin, an FDA-approved diabetes drug, promotes proliferation, self-renewal, and differentiation of adult neural precursors (NPCs). Specifically, we show that metformin enhances adult NPC proliferation and self-renewal dependent upon the p53 family member and transcription factor TAp73, while it promotes neuronal differentiation of these cells by activating the AMPK-aPKC-CBP pathway. Thus, metformin represents an optimal candidate neuro-regenerative agent that is capable of not only expanding the adult NPC population but also subsequently driving them toward neuronal differentiation by activating two distinct molecular pathways.

## INTRODUCTION

The observation of adult neural stem cells in the mammalian brain (Imayoshi et al., 2008; Reynolds and Weiss, 1992; Zhao et al., 2008) suggested that these stem cells could be mobilized for the repair of the injured or degenerating brain. A growing body of literature shows that adult neural stem cells are recruited in response to neural injury or degeneration, representing an attempt at endogenous repair (Kernie and Parent, 2010; Mitchell et al., 2004). However, this level of endogenous repair was not sufficient to repair the damaged brain. Thus, extensive efforts are underway to harness endogenous neural precursor cells (NPCs) as a novel regenerative therapeutic strategy to treat neural injury or brain degeneration. The recruitment of endogenous adult NPCs involves stimulation of multiple stages of adult NPC development, including proliferation, self-renewal, and differentiation. Thus, an optimal regenerative strategy would stimulate both proliferation/self-renewal and neuronal differentiation in order to generate sufficient numbers of new neurons to replace those lost after brain injury or degeneration.

Metformin, an FDA (Food and Drug Administration)-approved diabetes drug, was recently shown to promote adult neurogenesis under both physiological and pathological conditions in vivo (Liu et al., 2014; Jin et al., 2014; Wang et al., 2012). However, metformin has multiple molecular actions (Pernicova and Korbonits, 2014), and it is still not clear which ones are important for its neural effects. For example, metformin activates atypical protein kinase C (aPKC)-mediated CREB-binding protein (CBP) phosphorylation to regulate gluconeogenic gene expression in liver cells and enhance embryonic murine and human NPC differentiation (He et al., 2009; Wang et al., 2012). Moreover, metformin increases the levels of the p53 family member transcription factor TAp73 in cancer cells (Engelmann et al., 2015; Rosenbluth et al., 2008), and TAp73 is essential for adult NPC self-renewal and proliferation (Fujitani et al., 2010), suggesting that this protein might also be important for metformin's effects in the brain.

Here, we show that metformin treatment enhances both proliferation/self-renewal and neuronal differentiation of adult NPCs by activating two different molecular pathways. Metformin increases adult NPC proliferation/self-renewal

via TAp73 while it promotes neuronal differentiation by activating the AMPK-aPKC-CBP pathway. Thus, metformin represents an optimal neuroregenerative agent to recruit endogenous neural stem cells to replace the loss of neural cells after brain injury and degeneration.

## RESULTS

### Metformin Enhances Proliferation/Self-Renewal and Neuronal Differentiation of Adult NPCs

Previously, we showed that metformin, an FDA-approved diabetes drug, promotes embryonic murine and human NPC differentiation in culture and adult neurogenesis in vivo (Wang et al., 2012). To ask whether metformin acts on multiple stages of adult neural precursor development to increase adult murine neurogenesis in vivo, we used adult subventricular zone (SVZ) neurosphere cultures as a model system to assess functional roles of metformin in regulating the proliferation, self-renewal, and neuronal differentiation of adult NPCs. First, we examined primary neurosphere formation. Metformin (500 nM), added to freshly isolated SVZ NPCs for 6 days, robustly increased the number and size of primary neurospheres, indicators of self-renewal and proliferation, respectively (Figures 1A–1C). Metformin treatment of primary neurospheres also enhanced the number and size of secondary and tertiary neurospheres that were passaged every 4 days in the absence of metformin (Figures 1D and 1E), indicating that transient metformin treatment produces sustained NPC self-renewal and proliferation responses. Second, we treated dissociated NPCs derived from primary neurospheres with metformin (1 μM) for 7 days. Metformin treatment significantly increased the number of secondary neurospheres (Figure 1F), again indicating augmented self-renewing ability. Third, metformin significantly enhanced neuronal differentiation (Figures 1G and 1H), as we have previously published for embryonic NPCs (Wang et al., 2012). Finally, we examined the effect of metformin on adult NPC survival. Monolayer-cultured adult NPCs were treated with metformin (500 nM or 1 μM) for 2 days and assessed for cleaved caspase-3 by immunostaining and condensed nuclei. NPCs underwent apoptosis at a relatively low rate (~5%) in both vehicle (PBS) and metformin-treated cultures at 500 nM, while apoptosis was decreased to ~3% by 1 μM metformin (Figure S1). Therefore, inhibition of apoptosis was most likely not a major contributor to the robust increase in the number and size of adult neurospheres induced by metformin treatment.

We performed similar experiments in vivo, treating adult mice with metformin intraperitoneally (i.p.; 200 mg/kg) for 7 days, followed by a single bromodeoxyuridine (BrdU) injection (i.p., 100 mg/kg to mark proliferating NPCs). Mice were sacrificed 24 hr later to assess the total number of BrdU-positive cells, which represent cycling NPCs. Metformin treatment in vivo significantly expanded the BrdU-positive NPC populations in the SVZ (Figures 1I and 1J), consistent with the SVZ neurosphere data. We also assessed the number of BrdU-positive cells in the subgranular zone (SGZ) of the dentate gyrus, the other major neurogenic region in the adult brain. As seen in the SVZ, metformin increased the total relative number of BrdU-positive cells in the SGZ (Figures 1K and 1L). Thus, metformin promotes multiple stages of adult NPC development including proliferation, self-renewal, and neuronal differentiation.

### TAp73 Is Required for Metformin-Enhanced Proliferation and Self-Renewal of Adult NPCs

Based on our previous work showing that aPKC-mediated CBP phosphorylation at S436 is required for metformin-stimulated differentiation of embryonic NPCs, we asked whether this pathway was also involved in metformin-induced proliferation and/or self-renewal of adult NPCs. To do this, we performed adult neurosphere culture of wild-type (WT) mice in the presence of a pan-PKC inhibitor, chelerythrine, and adult neurosphere culture from a phospho-mutant CBPS436A knockin (*CBPS436A-KI*) mouse strain, in which the aPKC-CBP pathway is deficient due to the change of the serine (S) 436 residue to alanine (A) in the aPKC phosphorylation site of the CBP allele (Zhou et al., 2004). Quantification showed that chelerythrine did not block the increase in neurosphere formation induced by metformin (Figures 2A and 2B). Consistent with these data, metformin enhanced the number of primary and secondary neurospheres cultured from adult *CBPS436A-KI* as well as WT mice (Figures 2C and 2D).

These data suggest that metformin must act through a second pathway to enhance the proliferation and self-renewal of adult NPCs. In this regard, in cancer cells, metformin increases TAp73 levels (Engelmann et al., 2015; Rosenbluth et al., 2008), and TAp73 is essential for adult NPC self-renewal and proliferation (Fujitani et al., 2010). Therefore, we asked whether metformin might act via TAp73 to promote NPC self-renewal and proliferation. First, we determined whether metformin increased TAp73 mRNA levels in NPCs as it does in cancer cells. qRT-PCR of primary adult neurosphere mRNA showed that *TAp73* expression levels were significantly increased by metformin treatment (Figure 2E). Next, we investigated whether TAp73 was responsible for the metformin-induced increase in NPC proliferation and self-renewal. To assess this, we isolated adult SVZ neurospheres from mice where TAp73 is specifically knocked out (Tomasini et al., 2008) and treated them for 6 days with 500 nM metformin. Metformin enhanced the number of neurosphere-initiating cells