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BMP and TGF-β pathway mediators are critical upstream regulators of Wnt signaling during midbrain dopamine differentiation in human pluripotent stem cells

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ABSTRACT

Although many laboratories currently use small molecule inhibitors of the BMP (Dorsomorphin/DM) and TGF-β (SB431542/SB) signaling pathways in protocols to generate midbrain dopamine (mDA) neurons from hES and hiPS cells, until now, these substances have not been thought to play a role in the mDA differentiation process. We report here that the transient inhibition of constitutive BMP (pSMADs 1, 5, 8) signaling, either alone or in combination with TGF-β inhibition (pSMADs 2, 3), is critically important in the upstream regulation of Wnt1-Lmx1a signaling in mDA progenitors. We postulate that the mechanism via which DM or DM/SB mediates these effects involves the up-regulation in SMAD-interacting protein 1 (SIP1), which results in greater repression of the Wnt antagonist, secreted frizzled related protein 1 (Sfrp1) in stem cells. Accordingly, knockdown of SIP1 reverses the inductive effects of DM/SB on mDA differentiation while Sfrp1 knockdown/inhibition mimics DM/SB. The rise in Wnt1-Lmx1a levels in SMAD-inhibited cultures is, however, accompanied by a reciprocal down-regulation in SHH-Foxa2 levels leading to the generation of few TH+ neurons that co-express Foxa2. If however, exogenous SHH/FGF8 is added along with SMAD inhibitors, equilibrium in these two important pathways is achieved such that authentic (Lmx1a+Foxa2+TH+) mDA neuron differentiation is promoted while alternate cell fates are suppressed in stem cell cultures. These data indicate that activators/inhibitors of BMP and TGF-β signaling play a critical upstream regulatory role in the mDA differentiation process in human pluripotent stem cells.

Introduction

Cell replacement therapy remains a potentially important treatment strategy to replace the dead or dying midbrain dopamine (mDA) neurons that underlie Parkinson's Disease (PD). The success of this approach, however, greatly depends upon the discovery of an abundant source of cells capable of mDAergic function in the brain. Currently, pluripotent stem cells, either human embryonic stem cells (hES cells) or human induced pluripotent stem cells (hiPS cells) remain the most promising source of cells capable of differentiating into mDA neurons (Kim et al., 2002; Ben-Hur et al., 2004; Yang et al., 2004; Arenas, 2005; Hedlund et al., 2008; Cai et al., 2009, 2010; Friling et al., 2009; Lee et al., 2010). Understanding the mechanism underlying dopaminergic differentiation from pluripotent stem cells is key to successfully obtaining large numbers of transplantable cells for PD cell replacement therapy.

This endeavor has been greatly facilitated by studies examining similar mDA differentiation processes in the developing mouse midbrain (Ye et al., 1998; Arenas, 2002; Simon and Bhatt, 2003; Andersson et al., 2006; Prakash and Wurst, 2006; Prakash et al., 2006; Pollard et al., 2008; Joksimovic et al., 2009; Nakatani et al., 2010; Zhang and Zhang, 2010). In brief, development of mouse mDA neurons depends upon spatial and temporal differentiation cues derived from two key brain centers, the mid-hindbrain isthmus and the midbrain floor plate (Roussa and Krieglstein, 2004). The glycoprotein Sonic hedgehog (SHH) which is secreted by floor plate cells is thought to regulate dorsal–ventral patterning (Ye et al., 1998; Blaess et al., 2006) along with FGF8 while positioning along the anterior–posterior axis is mediated by the proto-oncoprotein Wnt1 derived from isthmus cells (Prakash and Wurst, 2006; Prakash et al., 2006). These secreted factors act by inducing expression of complex interrelated transcriptional cascades which are thought to specify an mDA fate in midbrain neuroepithelial cells (Chung et al., 2009;

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Lin et al., 2009). Key among these is the gene for LIM homeobox transcription factor 1 alpha (Lmx1a) which lies downstream of Wnt (Andersson et al., 2006; Cai et al., 2009, 2010; Chung et al., 2009; Friling et al., 2009). The transcriptional repressor or homeobox protein Msx1 and bicoid-like protein Otx2, promoting neuronal differentiation (via transcription factor Ngn2) and directly regulating the mDA transcription factors Nurr1 and Pitx3 while suppressing alternative cell fates (Andersson et al., 2006; Kittappa et al., 2007). Working coordinately with the floor plate forkhead transcription factors (Foxa1/2) which lie downstream of SHH, Lmx1 is thought to commit mouse floor plate cells to an mDA fate (Kittappa et al., 2007; Chung et al., 2009; Lin et al., 2009; Lee et al., 2010; Nakatani et al., 2010).

Over the last decade, significant strides have been made in developing tissue culture protocols that recapitulate the mDA differentiation process in hES and hiPS cell cultures (Cai et al., 2009, 2010; Chung et al., 2009; Friling et al., 2009; Cooper et al., 2010; Fasano et al., 2010; Nakatani et al., 2010). Most of these employ a 5-stage protocol that moves cells from the undifferentiated state, through pseudo-gastrulation in the embryoid body (EB) to mDA committed neural progenitors (hNPs) and finally into mDA neurons. However, recently, many labs studying hES and hiPS cells have moved away from EBs to monolayer cultures which use small molecule inhibitors of BMP/TGF-β signaling in their media formulations to enhance generation of neural progenitors and neurons by inhibiting mesenchymal differentiation (Chambers et al., 2009; Denham and Dottori, 2009). While a number of labs report the differentiation of mDA neurons in these monolayer cultures (Jaeger et al., 2011; Kim et al., 2011; Kriks et al., 2011; Vogt et al., 2011; Lipchina et al., 2012; Nefzger et al., 2012; Xi et al., 2012), there have been no systematic studies of the effects of BMP/TGF-β inhibitors specifically on the mDA differentiation process.

In general, the superfamily of TGF-β ligands (BMPs, GDFs, activin, nodal, etc.) are thought to mediate their effects by binding specific receptors which phosphorylate SMADs and co-SMADs to form complexes that move to the nucleus where they bind transcription factor promoters. Inhibitors such as DM bind BMP type I receptors ALK2, ALK3 and ALK6 to block phosphorylation of SMADs 1,5,8 (Yu et al., 2008). SB inhibits the activin type I receptor ALK5, the TGFβR1 receptor ALK4 and the nodal type I receptor ALK7 which phosphorylate SMADs 2 and 3 (Inman and Hill, 2002). Whether SMAD inhibition by small molecule BMP and/or TGF-β inhibitors alters mDA differentiation and whether it does so by affecting specific transcription factors etc. remains to be established.

In this paper, we will show evidence that transient inhibition of the constitutive BMP pathway, either alone or in combination with TGF-β inhibition, is critical to the upstream regulation of the SMAD-interacting protein 1 (SIP1) and its downstream effector secreted frizzled related protein 1 (Sfrp1) and their reciprocal regulation of Wnt1-Lmx1a and Shh-FoxA2 signaling during mDA differentiation in stem cell cultures. However, generating authentic mDA neurons is achieved only when a proper balance between Wnt and SHH pathways is attained which requires both SMAD inhibition and exogenous SHH/FGF8.

Results

In the last year, in addition to our usual EB method of differentiating mDA neurons from hES/hiPS cells, cells were also differentiated using a simplified monolayer method (Fig. 1A). This was in part made possible by the discovery that the rho-associated kinase (ROCK) inhibitor, Y-27632, markedly diminishes dissociation-induced apoptosis in stem cells (Watanabe et al., 2007), allowing us to proceed directly from undifferentiated cell colonies to mDA differentiation while omitting the EB step.

Curiously, when hNPs were generated in monolayer cultures, we found that some hNPs expressed Lmx1a but rarely went on to develop into tyrosine hydroxylase (TH)-expressing neurons at stage 5. Instead, they appeared to be 'trapped' at the mDA specification step (Fig. 1B). However, when both the BMP inhibitor, Dorsomorphin (DM) and the TGF-β inhibitor, SB431542 (SB) were added, we found a dramatic rise in Lmx1a expression in NPs as well as a marked amplification in the number of TH+ mDA neurons, disproportionate to the increase in nestin+ progenitors and β-III tubulin+ (β-III tub) neurons observed in the same cultures (Fig. 1B,C). Likewise, when EB cultures were treated with DM/SB, there was a significant increase in Lmx1a and TH over nestin and β-III tub expression (Fig. 1B,C). Taken together, these results suggested that while DM/SB modestly increases NP and neuron production in monolayer cultures, it greatly increases the proportion of those cells that are mDA-specified and that go on to become TH+ neurons.

We next investigated the mechanism via which BMP/TGF-β inhibitors of specific receptor SMADs exerted their effects on mDA differentiation. Western analysis of hES cells maintained in basal growth media (control cultures) exhibited moderate levels of pSMADs 1, 5, 8 and pSMADs 2, 3 (Fig. 2A, C). However, constitutive BMP signaling was nearly totally blocked after treatment (stage 2) with highly specific BMP pathway inhibitor, DM (Fig. 2A, C). In contrast to DM, 10 μM SB was a relatively ineffectual inhibitor of the TGF-β pathway, only partially blocking the formation of pSMADs 2, 3 in stage 2 (Fig. 2A, C). After removal of SMAD inhibitors, phosphorylation of all SMADs was restored to near normal levels in stage 3.

To identify potential downstream molecular targets of BMP/TGF-β inhibitors, we used human PCR arrays (Qiagen PAHS-047Z — stem cell signaling) or (Qiagen PAHS-035Z — BMP/TGF-β signaling pathway) to compare control and DM/SB-treated monolayer cultures. While a number of genes were induced by DM/SB treatment, only those that were increased at least 5-fold upon treatment were verified by qPCR (Suppl. Fig. 1). Of that group, we found that inhibition of SMAD signaling in both EB and monolayer cultures caused a dramatic rise in the levels of the transcription factor, SMAD-interacting protein 1 (SIP1, also known as Zinc finger E-box-binding homeobox 2 or ZEB2). Interestingly, SIP1 levels were also elevated in untreated EB cultures compared to untreated monolayers, suggesting that the same factors may have been involved in mediating mDA differentiation in EB cultures even in the absence of DM/SB supplementation, possibly as a result of endogenous BMP/TGF-β inhibitors (ie. noggin) (Chambers et al., 2009; Krause et al., 2011).

An important confirmation of SIP1's role in mDA specification and differentiation was provided by SIP1 knockdown experiments. In these studies, SIP1 shRNA and control (empty and scramble) vectors were transfected into undifferentiated stem cells. Following puromycin selection and subsequent differentiation, qPCR analysis revealed significant knockdown in SIP1 transcripts, and importantly, a reduction in Lmx1a in stage 4 hNPs and TH in stage 4/5 neurons, without a change in nestin or β-III tub expression (Fig. 3A). Cleaved caspase 3 protein was not increased in SIP1 knockdown cultures (Fig. 3B), indicating that the decrease in Lmx1a and TH was not due to enhanced toxicity/cell death from genetic engineering. These data demonstrate that SIP1 knockdown results in decreased mDA specification and differentiation without altering neurogenesis, suggesting that the two developmental processes are likely mediated by different pathways acting downstream of DM/SB. Moreover, these data further suggest that constitutive SIP1 levels normally hold in check Wnt1-Lmx1a-TH expression in stem cells, and that by