FACULTY OF MEDICINE
Lund University

Lund, Sweden

Dear Professor ZZZ,

We are very grateful that you consider a second revised edition of our manuscript 123123123 entitled "....." for publication in *Journal of Neuroscience*. We are particularly grateful that you allowed us more time to perform all the experiments the reviewers suggested.

Based on the helpful comments of the referees, we have now re-structured the manuscript and described in a (hopefully) clearer way all the data we found.

Thus, we have completely re-organized the manuscript in a manner that we hope will make it more accessible to the reader. As is evident from the new subheadings, now we first describe the effects of the four transcription factors on cell maturation, and focus on neuronal and glial differentiation. We then describe the effects of simultaneously overexpressing two of the genes on neuronal and glial differentiation. Next we report the effects of the four genes on cell-cycle exit, proliferation and cell-death. In the subsequent sections we described the impact of overexpressing the transcription factors, either alone or in dual combinations, on differentiation of rat progenitors into dopaminergic neurons. Finally, we report that overexpression of Lmx1a, but not Msx1, increases dopaminergic differentiation in a human midbrain progenitor cell line.

While reviewer n°2 was completely satisfied by our previous round of revisions, reviewer n°1 was mainly concerned about the following three points:

1. Whether we could reproduce data reported by XXX et al (2006).
2. The maturation state of Ngn2 and Pitx3-overexpressing cells.
3. The effects of dual transgene delivery on glial differentiation.

We have performed all the experiments and addressed all the issues raised by reviewer n°1. We describe our revisions in detail below, and hope that the manuscript now is acceptable for publication.

We would prefer that our manuscript is not reviewed by referees from the XYZ institute (.....). Because they published the pro-dopaminergic effect of Lmx1a (XXX et al 2006) and acquired intellectual property rights based on their results, they have a conflict of interest. Their report was published in *yyy* in January 2006, and described Lmx1a and Msx1 as key transcription factors in dopaminergic neuron development. They reported that mouse embryonic stem cells (mESC)

overexpressing Lmx1a differentiate into dopamine neurons in the presence of Sonic hedgehog (SHH) and FGF8. During the past 6 months, we have tried to replicate these results in three separate sets of experiments:

1- We used a lentiviral vector to express human Lmx1a under the Nestin Enhancer-TK promoter in mESCs. In analogy with the study by XXX et al, we supplemented the medium with bFGF, SHH and FGF8. Unfortunately, these mESCs did not differentiate into dopaminergic neurons.

2- We also used two retroviruses that we originally generated for the present study on ventral midbrain progenitors. Thus, during the past 6 months we transduced mESCs with Lmx1a and Msx1, coupled to a GFP reporter. We grew the cells in the presence of bFGF, SHH and FGF8. Again, the treatments did not result in the induction of dopaminergic neurons. In this letter, we present the results of the key immunostains we performed on the transduced mESCs (Fig. 1). Both the transgene (Lmx1a or Msx1) and the reporter gene eGFP were highly expressed, and the transduced cells were nestin-positive. Unfortunately, the cells rarely became for β-III-tubulin-positive neurons or TH-immunopositive.

3- We also performed one set of experiments on mESC that were co-cultured with PA6 stromal cells, and treated as described above (both condition 1 and 2). It is well established in the literature that PA6 cells induce a high degree of dopaminergic differentiation in ESCs. We did not observe any increase in the proportion of cells that became TH-immunoreactive when we overexpressed either Lmx1a or Msx1.

In summary, it is with frustration that we have to conclude that none of the above mentioned conditions that all involve overexpression of Lmx1a or Msx1 promote the differentiation of mESC into TH-expressing dopaminergic neurons.

Following the publication of the *xyz* paper in 2006, there are no published original articles describing the effects of Lmx1a and Msx1 on stem cells. We are aware of discussions at conferences and workshops that other groups have also not been able to replicate the impressive findings of the original XXX et al 2006 paper.

We do not, however, want to include the studies we performed on mESC during the past 6 months in our manuscript. We feel it distracts attention from the main message, which is to report the effects of the four transcription factors we studied on neural progenitors. We simply do not wish only to overly stress the absence of pro-dopaminergic effects of Lmx1a and Msx1 by including the new mESC data in this publication. We are convinced that our work on ventral midbrain progenitors is very important already as it stands. We state this information to you in this letter, just to emphasize that we have taken the requests of the initial reviewers very seriously.

We are certain that the scientific community will receive our data with great interest. Notably, our study is also the first to examine the effects of Lmx1a and Msx1, in neural stem cells, and the first to investigate their effects in human cells. Furthermore, we describe in greater detail than ever done before the developmental effects of Pitx3 and Nurr1 in midbrain progenitors. We have performed extensive experiments, which

we believe are of very high quality. The data are interesting from a developmental biology standpoint, and also highly relevant to research aimed at developing a cell therapy for Parkinson's disease

We look forward to your response.

Yours sincerely,

ZYX, PhD YZX, PhD, MD

PHOTO IN HERE

FACULTY OF MEDICINE
Lund University

Lund, Sweden

Answers to Reviewer comments.

First, we want to draw the attention of the reviewers to the facts that we have re-organized the manuscript in a manner that we hope will make it more accessible to the reader. As is evident from the new subheadings, now we first describe the effects of the four transcription factors on cell maturation, and focus on neuronal and glial differentiation. We then describe the effects of overexpressing two of the genes simultaneously on neuronal and glial differentiation. Next we report the effects of the four genes on cell-cycle exit, proliferation and cell-death. In the subsequent sections we described the impact of overexpressing the transcription factors, either alone or in dual combinations, on differentiation of rat progenitors into dopaminergic neurons. Finally, we report that overexpression of Lmx1a, but not of Msx1, increases dopaminergic differentiation in a human midbrain progenitor cell line.

Comments from the reviewers are in **bold**, our reply are in normal text and our changes in the manuscript in *italics.*

Reviewer: 1

Major Comments

1. One of the major points of this manuscript is that controlled expression of transcription factors in mESCs (XXXX et al., 2006) differs from overexpression of transcriptions factors in E14.5 midbrain cultures in that mESCs possess greater plasticity to generate midbrain dopaminergic neurons. However, little is made of this comparison. It would be nice to see, in the hands of the authors, that overexpression of midbrain DA neuron transcription factors (i.e., Lmx1a) in mESCs does indeed result in midbrain dopaminergic neurons. This would provide a positive control for the reported experiments in neural progenitors.

We appreciate the comment of the reviewer. We agree that we cannot draw a definite conclusion that mESCs possess a greater plasticity than E14.5 midbrain progenitors when it comes to their ability to make dopamine neurons. For this we would need additional data, and should not only refer to the previous publication. Indeed, it would be valuable to have data showing that Lmx1a overexpression generates dopaminergic neurons from mESC using the exact same, or at least closely related, reagents (viral vectors, growth factors, TH antibody, etc) as we did in our experiments with the midbrain progenitors. Thus we have tried to reproduce the results previously reported by XXXX and co-workers using three different paradigms on mESC cultures (we have performed two independent experiments for each of the conditions described below:

1- We used a lentiviral vector to express human Lmx1a under the Nestin Enhancer-TK promoter in mESCs. In analogy with the study by XXXX et al, we supplemented the medium with bFGF, SHH and FGF8. Unfortunately, these mESCs did not differentiate into dopaminergic neurons.

2- We also used two retroviruses that we originally generated for the present study on ventral midbrain progenitors. Thus, during the past 6 months we transduced mESCs with Lmx1a and Msx1, coupled to a GFP reporter. We grew the cells in the presence of bFGF, SHH and FGF8. Again, the treatments did not result in the induction of dopaminergic neurons. In this letter, we present the results of the key immunostains we performed on the transduced mESCs (Fig. 1). Both the transgene (Lmx1a or Msx1) and the reporter gene eGFP were highly expressed, and the transduced cells were nestin-positive. Unfortunately, the cells rarely became for b-III-tubulin-positive neurons or TH-immunopositive.

3- We also performed one set of experiments on mESC that were co-cultured with PA6 stromal cells, and treated as described above (both condition 1 and 2). It is well established in the literature that PA6 cells induce a high degree of dopaminergic differentiation in ESCs. We did not observe any increase in the proportion of cells that became TH-immunoreactive when we overexpressed either Lmx1a or Msx1.

Unfortunately, it is with frustration that we observed that, in none of the aforementioned conditions, neither Lmx1a nor Msx1 (in combination with SHH, FGF8 and FGF2 for all three conditions described above) led to the formation of TH-expressing dopaminergic neurons.

Naturally this raises the question whether there is something wrong with our technique or reagents. For several reasons, we do not believe this is the case. *First*, we demonstrated that all our vectors work. Thus, the transgenes and reporter genes were expressed, as revealed very clearly by immunocytochemistry and RT-PCR for the respective proteins. *Second*, in the different gene transduction experiments we observed effects on cell differentiation, cell cycle exit, etc, indicating that our vectors were functional. Several of these results are supported by those previously described in the literature. *Third*, for all constructs we either sequenced them ourselves or received them directly from well-established research groups that have used them over several years in their studies. This adds further support to the credibility of our findings.

Despite the extensive work and effort we have invested into the experiments on mESC during the past 6 months, we do not want to include the studies we performed in our manuscript. We feel it distracts attention from the main message, which is to report the effects of the four transcription factors we studied on midbrain progenitors. We simply do not wish only to overly stress

the absence of pro-dopaminergic effects of Lmx1a and Msx1 by including the new mESC data in this publication.

As a result of our negative findings in mESC, we have downplayed the idea in the text (page 4) that "…**mESCs possess greater plasticity to generate midbrain dopaminergic neurons"**, which was the initial concern of the reviewer. Therefore we hope the revision we have made in the text is now satisfactory to Reviewer no. 1.

---Fig 1 in here --

Figure 1. Immunocytochemistry performed on EB5 mESC that have been transduced with retroLmx1aIRES2eGFP or retroMsx1IRES2eGFP. Transduced cells mainly expressed the neural progenitor marker Nestin (upper panels). Transduced cells express both the transgene (Lmx1a or Msx1) as well as the reported gene (eGFP) (central panels). Neither Lmx1a nor Msx1 induced neuronal maturation of the mESC transduced cells, even after 16 days differentiation in vitro (lower panels). Neither Lmx1a nor Msx1 induced dopaminergic differentiation of the mESC transduced cells, even after 20 days differentiation in vitro (data not shown as TH was only rarely detected in cultures). The mESC line was kindly provided by Dr. YYYYY, ZZZ.

2. Sox2 is the only marker used to determine progenitor status. Was nestin also downregulated following retroviral infection with Ngn2 and Pitx3?

We agree with the referee that this information is important. We initially only used Sox2 because it is a more specific neural progenitor marker than nestin. Nevertheless, we agree that the addition of Nestin immunostaining is valuable. Therefore, as suggested by the reviewer, we have now performed immunocytochemistry for nestin on Ngn2- and Pitx3-transduced cultures. In agreement with Sox2-immunostaining, we found that nestin was distinctly downregulated (>95% of the cells negative) when one of these transgenes was expressed.
This information has been added in the manuscript on page 13. The text now reads:

Similar results were obtained using the neural progenitor marker Nestin. Less than 5% of the Pitx3-GFP cells and Ngn2-GFP cells were co-labeled with Nestin (Supplementary Fig. 1A and B).
We also provide two pictures showing the results of the Nestin immunocytochemistry we performed, in supplementary figure 1 A and B.

3. Based on the studies to determine whether progenitors were becoming post-mitotic following introduction of factors, cell cycle exit was examined. However, the results obtained appear to contradict the results found in other studies. For example, Pitx3 maintained cells in a proliferative state, but stopped expression Sox2. Conversely, Lmx1a showed low levels of

CldU incorporation (very significantly lower than control) but high levels of Sox2 (similar levels to the control). These observations need to be discussed.

We agree with the reviewer that the observations that Pitx3-transduced cells downregulated Sox2 and still continued to proliferate were unexpected. We have now also performed Nestin immunocytochemistry on Pitx3-transduced cells and observed that this marker too is downregulated. The downregulation of Sox2 and Nestin clearly indicates that Pitx3-transduced cells are no longer in immature neural progenitors. The cells did, however, not express neuronal, astrocytic or oligodendrocytic markers. Instead they continued to divide, as mentioned by the reviewer. As a consequence of the remarks of this reviewer, we have now revised the section in the discussion on Pitx3 (shown below):

Pitx3 is involved in the maintenance of the dopaminergic phenotype

Pitx3 plays a role during late stages of the embryonic development of dopaminergic midbrain neurons {.....}. Pitx3 expression commences when AADC-positive dopaminergic neuroblasts have migrated to the ventral part of the central midbrain. This is independent from the Nurr1-mediated transcriptional cascade, because Nurr1 knock-out mice still express Pitx3 and AADC {.....). The primary role of Pitx3 in the midbrain is to promote the survival of dopaminergic neurons, once they have formed and migrated ventrally. We show that Pitx3-transduced cells stopped expressing Sox2 and nestin, but did not adopt a neuronal or glial morphology. Interestingly, despite downregulating these markers for immature proliferating neural cells, Pitx3-transduced progenitors continued to divide. During normal development of midbrain dopaminergic neurons, Pitx3 appears after the dopaminergic neuroblasts have become post-mitotic. Thus, effects of Pitx3 overexpression on dividing midbrain cells cannot be predicted from normal midbrain development. Indeed, Pitx3, which is normally expressed in different tissues, might play diverse roles depending on when in the cell cycle it is expressed. In the ventral midbrain, Ngn2 is probably the most potent inducer of cell cycle exit and is expressed prior to Pitx3 in dopaminergic neurons. In our experiments, we overexpressed Pitx3 in cells that had not yet expressed Ngn2. Therefore, the action of Pitx3 in our paradigm differed from that seen in normal developing midbrain. Thus, it downregulated proteins expressed in immature cells (Sox2 and nestin) without inducing cell cycle exit and maturation into neurons or astrocytes. Consequently, our Pitx3-transduced cells could not differentiate into dopaminergic neurons. This is consistent with a recent study showing that mouse and human ESCs overexpressing Pitx3 do not generate dopaminergic neurons {.....}. On the other hand, in mouse ESCs Pitx3 can maintain already induced TH and DAT expression {....}.

A recent study showed that signals present in earlier stages of midbrain development are crucial for E14 midbrain-derived neurospheres to develop into dopaminergic neurons, and expression of Pitx3 can support their survival. When Pitx3-transduced E14 midbrain-derived neurospheres were co-cultured with E11 midbrain explants, an increased number of TH-expressing neurons appeared in the neurospheres (.....). Overexpression of Pitx3 in neurospheres co-cultured together with cortical tissue did not result in an increase the number of TH-positive neurons, demonstrating that the inductive effect is specific to midbrain tissue. Thus, in this paradigm Pitx3 has a unique role in maintaining dopaminergic neurons and inductive signals from early midbrain development are required to induce dopaminergic differentiation in neurospheres derived from late midbrain progenitors.

Concerning the low levels of CldU incorporation in Lmx1a transduced cells and high levels of Sox2, we have extended our discussion on these results in the discussion, as shown here:

The Lmx1a-transduced cells incorporated less CldU than controls or those transduced with Msx1, or Pitx3. However, CldU incorporation is not completely shut off. Thus, the Lmx1a-transduced cells continue to proliferate, albeit at a lower rate. These cells curiously also express high levels of the immature markers Sox2 and nestin, as well as the mature neuronal marker βIII-tubulin. This suggests that at least two pathways are affected by Lmx1a overexpression, producing a marker expression profile representing proliferating neuroblasts.

In addition, how can it be reconciled that Lmx1a caused a significant exit from the cell cycle, but yet a trend for increased numbers of cells in the culture (pg.18).

We have chosen not to comment on this in the manuscript because the trend for an increased number of cells in the Lmx1a treated cultures was not statistically significant. Therefore the observed trend may just have been the result of chance variation, and not a true biological effect of the transgene. Nevertheless, we are happy to discuss the trend for an increased number of cells in this response to the Reviewer. Should this trend actually be a true effect of Lmx1a overexpression, two potential mechanisms come into mind. Because Lmx1a overexpression promotes cell cycle exit at a statistically significant level, it is conceivable that the effect of the transcription factor is not uniform on all cells because they are a heterogeneous population at the time of transduction. Thus, some of the transduced neuroblasts become post-mitotic and other retain the ability to proliferate. Another plausible explanation is that Lmx1a overexpression not only promotes cell cycle exit, but also reduces cell death, leading to the trend for an increased number of cells in the cultures.

Also, the incorrect figure is referenced on pg. 18 (fig. 4, not fig. 5, for cell cycle data).

We thank the referee for noting this mistake. We have now corrected this.

4. The co-expression experiments are a good addition; however, little information is presented regarding what they become. Is their phenotype similar to induction with one of the transcription factors alone?

We agree with the reviewer that information on the fate of the double transduced progenitors is valuable. We thank the referee for raising this important issue and have now performed a series of additional immunocytochemical stainings to examine whether the dual transgene overexpression promotes glial or neuronal differentiation. We have added some sentences on this topic (p15-16), and a new figure (figure 4) illustrating results from the triple labeling immunocytochemistry.

The new section in the Results now reads:

Effect of dual retroviral transgene delivery on neuronal and glial differentiation

We next studied whether Lmx1a, Msx1, Ngn2 and Pitx3 can synergistically induce neuronal or glial differentiation of ventral midbrain progenitors. Lmx1a is the first of the four genes of interest that is expressed during dopamine neuron development. Therefore, in an attempt to promote the generation of midbrain-derived neurons, we overexpressed Lmx1a combined with each of the

three remaining genes. To this aim, we generated a retroviral vector capable of expressing DsRed2 reporter gene, in which we subcloned Lmx1a. In an initial experiment we evaluated the efficacy of the dual transgene delivery. For this purpose, we transduced the ventral midbrain progenitors using control retroviruses that only drove the expression of either a DsRed2 or eGFP reporter gene. One day after dual transduction, the cells started to express both reporter genes (Fig. 4A1-A4), demonstrating that the procedure had worked. At four days, a majority of the cells had migrated out of the core neurosphere and expressed both reporter genes. When we used an MOI of 1, between 30 and 50% of all cells co-expressed DsRed2 and eGFP.

We then examined the effects of simultaneously overexpressing two transcription factors. Eight days following dual transgene delivery, over 70% of Lmx1a:DsRed2/Ngn2:eGFP-overexpressing cells were MAP2-immunopositive (Fig. 4E1 and E2). This result is similar to that we obtained when we overexpressed Ngn2 alone (Fig. 2H and L). Thus, isolated Ngn2 overexpression is sufficient enough to promote neuronal differentiation. By contrast, neuronal differentiation was low when we combined Lmx1a with Msx1 (<6%) or Pitx3 (<5%). Indeed, neither dual overexpression of Lmx1a and Msx1 nor Lmx1a and Pitx3 increased neuronal differentiation, beyond what we had observed when only overexpressing Msx1 or Pitx3, respectively (Fig. 4C1, C2, G1 and G2). When we assessed glial differentiation, we found that almost none of the cells double transduced for the genes of interest expressed GFAP or CNPase (Fig. 4D1. D2, F1, F2, H1 and H2).

Taken together, these data demonstrate that co-transducing ventral mesencephalic progenitors with lmx1a combined with Ngn2, Msx1 or Pitx3 neither enhance neuronal differentiation beyond what is seen when transducing the cells with the transcription factors individually, nor promote glial differentiation.

In the discussion, it is pointed out that controlled expression of Lmx1a may be required for DA neuron generation. This is likely to be true. However, it is critical to determine whether the overexpression paradigm used here will actually work on mESCs.

Please see our response to comment no. 1 above. As we describe in detail above, during the past 6 months we have extensively tested Lmx1a overexpression in three different paradigms in mESCs and do not observe an increase in the number of TH expressing neurons. We do not, however, feel that the present manuscript is the right forum to discuss our new observations in mESCs, because we want to focus on the biological effects of the four transcription factors on rat midbrain progenitors and a human neural cell line.

Minor comments

1. The last sentence on pg. 12, " Since the single overexpression ." is worded in a misleading way and makes a simple point unclear to the reader.

We agree with the referee that the sentence was misleading. As we re-structured the manuscript, the sentence was deleted.

2. Fig. 1 legend. Where is information regarding data in H?

Thank you for pointing this out. The information was in the figure legend, however, we had forgotten to specifically attribute the letter H in brackets to refer it to. The mistake has been corrected.

The new sentence reads: *RT-PCR shows the ventral midbrain progenitors have the capability to differentiate into dopaminergic neurons regardless of Lmx1a transgene overepression (H).*

3. gliogenesis, not gliagenesis, Table 1: sense, not sens

Again, thank you for identifying these two typos. We have now corrected both.

Reviewer: 2

1. Ctlr should be Ctrl in some of the figures

Thank you for noting this. We have corrected this in all figures.

2. The abstract states that the human neural cells have a three-fold increase in TH neurons. First the authors did not use human neural stem cells, rather a cell line. Second, they admit that this only represented a small number (<3%) of dopamine neurons. This should be dampened down in the abstract to avoid confusion.

We totally agree with the referee that we had overemphasized our data on human NSC differentiation. We have therefore dampened-down the sentence in the abstract. The text has been rewritten both in the Abstract and Discussion and it now reads:

A- In the abstract (4): *Notably, the overexpression of Lmx1a, but not Msx1, in human neural progenitors increased the yield of tyrosine hydroxylase-immunoreactive cells by 3-fold.*

B- In the discussion (P13): *The proportion of TH-immunoreactive neurons increased 3-fold from 1% to 3%. Although the effect represents a proof-of–principle that Lmx1a can promote differentiation of human progenitors into dopaminergic neurons, the cells that expressed TH represented only a small minority of the cells that were transduced by the Lmx1a retrovirus. Indeed, the proportion of TH-immunoreactive cells was too low to be of practical importance in a neural transplantation context*