Reviewer's report

Title: The Src inhibitor dasatinib stimulates the differentiation of human bone marrow-derived mesenchymal stromal cells into osteoblasts

Version: 1 Date: 6 November 2009

Reviewer: Olivia Fromigue

Reviewer's report:

This manuscript entitled "The Src inhibitor dasatinib stimulates the differentiation of human bone marrow-derived mesenchymal stromal cells into osteoblasts" by Boufker et al. reports that the synthetic compound dasatinib favors the osteoblastic differentiation process of human mesenchymal cells in vitro. This suggests that in addition to its previously demonstrated anti-tumoral effects, dasatinib positive effects on bone formation may counterbalance bone loss in several diseases.

This paper points out the need to develop new therapy in order to promote bone formation. The Authors used human normal primary mesenchymal stem cells and standard methodologies. It is an in vitro study, therefore, it remains to be seen if such compound will also be effective in an animal model.

Major Compulsory Revisions:

- The conclusions are not fully supported by the data. 1/ The Authors claim on page 23 that "Our study reports an original dual effect of dasatinib on MSC at non toxic concentrations: (i) it is able to significantly stimulate the osteogenic differentiation of MSC". However, absolutely no significant effect was observed and reported on the different markers (ALP activity, calcium deposition or markers mRNA expression) when dasatinib was used alone. Only some transient stimulatory effects were observed in combination with DAG, on some markers. 2/ The Authors indicate that: "(ii) it can inhibit RANKL expression in undifferentiated MSC". The authors are not clear on the definition of undifferentiated cells. They should clarify if it corresponds to the early time points of culture (day 7) or to cells incubated in absence of DAG? In figure 8, dasatinib alone transiently reduced RANKL mRNA level at day 7 but did not modulate RANKL mRNA level thereafter. Dasatinib did not amplified the transient inhibitory effect of DAG. Is the difference between DAG and Dasatinib+DAG conditions on RANKL expression at day 7 (figure 8) non significative? The statistical analysis should be performed.

- In the discussion section (page 20), the Authors write "our experiments were performed with a non-toxic but effective concentration of dasatinib (10-8 M) able to inhibit Src kinase activity in accordance with a previous report showing inhibition of the kinase activity of purified Src protein with an IC50 of 3x10-9 M [27]" but this dosage concerns prostate cancer cells and not primary normal mesenchymal cells. The Authors should perform an evaluation of src phosphorylation status or activity in hMSC incubated in the presence or absence
of DAG and/or dasatinib. Although dasatinib is a known SRC family inhibitor, it may impact other signaling pathways, however no experiment was done to prove the specific inhibition of SRC pathway at 10nM.

- The in vitro model of hMSC culture used by the Authors requires DAG medium for induction of osteoblastic differentiation. It is reported in the literature that glucocorticoid receptor interacts and co-localizes with src (Matthews and coll., Mol Endocrinol 2008). The Authors should explore the effect of glucocorticoid supplementation on src activity first and also on dasatinib inhibitory effect of src activity in their hMSC system.

- The authors want to demonstrate that dasatinib stimulates the differentiation of hMCS into osteoblasts however in their experiments dasatinib exhibits no significant effect alone and only transiently amplifies DAG effect on hMSC leading to a goalless final effect.

- Glucocorticoids are reported to modulate cell proliferation. The authors should perform a dose-dependent effect of dasatinib on cell proliferation in DAG medium.

- The absence of calcium deposition (Figures 2A, 3A, 7A) in the control condition reported by the Authors is not surprising as beta-glycerophosphate (or another source of phosphate) supplementation in culture medium is an absolute requirement to allow extracellular matrix mineralization. It is thus difficult to evaluate the effect of dasatinib alone on ECM mineralization in these culture conditions. In that way, it is also difficult to compare dasatinib and DAG+dasatinib culture conditions in term of matrix mineralisation potency.

- Five of 8 figures described the culture model of hMSC in DAG medium. It did not represent a new model of hMSC’s osteoblastic differentiation. The positive effect of dexamethasone supplementation on human osteoprogenitor cells differentiation is known since several years (Cheng and coll., Endocrinology 1994; Cheng and coll., J Cell Biochem 1996; Fromigue and coll., cytokine 1997; Diefenderfer and coll., Connect Tissue Res 2003; Song and coll., J Orthop Res 2009).

Furthermore, the data presented in figures 3B and 7B are redundant with however some discrepancies: in control condition, ALP activity did not vary during the culture kinetic shown in figure 3B but was increased by about 6-7 fold from day 7 to day 21 in figure 7B. The Authors should then clarify the evolution of ALP activity in basal conditions.

Figures 4 and 5 and Table 2 are redundant but don't completely match each others. As for example: OSX is notified "0" at day 0 and "++" at day 7 on table 2 whereas its expression level is not significantly modulated on figure 4C. The Authors should clarify the real expression level of OSX.

In the same way, figures 4 and 8 did not match in term of mRNA expression levels under DAG treatments. AS for example, the Authors showed that BSP is weakly expressed at day 0 and markedly increased at day 7, 14 and 21 on fig 4C, whereas on figure 8, BSP mRNA level in DAG condition is not significantly
modulated compared to control condition. These results are then poorly convincing.

Minor Essential Revisions
- The Authors reported a weak and constant expression of Runx2 and type I Collagen in hMSC throughout the differentiation process (Figure 4B), as well as a very late induction of Osterix (day 21, figure 4C). These three gene products are known to be essential for osteoblastic cells, either as major transcription factors or as main extracellular matrix component. Although the Authors did not consider these genes as specific of some osteoblastic stages (figure 8), they should nevertheless determine the expression level of these markers in dasatinib-/+DAG conditions.

- Figure 2 : the cell density in DAG condition seems to be lower than in control conditions. Did DAG medium modify cell proliferation vs control medium? Moreover, can the Authors explain why the cells did not reach confluency, even after 21 days of culture?

- How did the Authors performe the optimization of PCR method? What are the saturation curves for each primer sets? From how many number of cycles did actin amplification saturate? How was determined the detection limit?

- The Authors should clarify if they presented results of one representative experiment or if they pooled the values. As for example, on figure 3 does "n=10" mean one replicate from 10 patient-derived samples or mean 10 replicates of the most representative sample?

Some SEM values are excessive (figures 3 and 7).

- Figure 4 : The Authors should clarify if ALP mRNA expression level was significantly increased at day 7 in comparison to day 0. Same remark for PTHr at day 14, OPN at day 7, and OSX at days 7 and 14. Statistical analyses should also be performed between each of the different time-points, and not only vs day 0.

- Figure 8 : The Authors should clarify if RANKL mRNA expression level was significantly decreased under DAG+dasatinib treatment compared to control condition at day 7?

- Page 11 : Since the activation step (15 min at 95°C) is realized only once before cycling, the sentence "Each cycle consisted of activation at 95°C for 15 min, denaturation at..." have to be corrected.

- The Authors should homogenize ALP activity units in figure legends and on figures : ALP activity should be expressed as "the p-nitrophenol concentration per 15 min for 10^4 cells" and not as "units/µl".

The calcium content measurement should be normalized to either cell number, total protein content ou total DNA content instead of "mg/dl".

Discretionary Revisions
Page 7: "cells were...replated at a density of 200 cells/cm² for all subsequent passages". Can the Authors clarify at which passage exactly were conducted the different experiments?

Page 8: What is the concentration of the dasatinib stock solution in ethanol?

Page 8: Did the Authors noticed a difference in cell response between young children (5 years old) and adults (up to 55 years)?

Page 8: "The osteogenic medium was changed weekly". The stability of ascorbic acid is low in culture media (t1/2 is about one day; Hata & Senoo, J Cell Physiol 1989). What about the stability of dasatinib in vitro?

Misspelling CBFA1 -> RUNX2 on figure 4B.

Level of interest: An article of limited interest

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests