Author’s response to reviews

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

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Version: 2 Date: 2 February 2010

Author's response to reviews: see over
Reviewer's report #1

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

**Version:** 1  **Date:** 31 October 2009

**Reviewer:** Dorit Naot

**Reviewer's report:**

The study investigates the effects of dasatinib on osteoblast differentiation. Examining the activity of dasatinib in bone is interesting and important and the biological question of the study is well-defined. The methods used in the study are described in detail, and the challenging experimental system of in vitro differentiation of human osteoblasts and formation of mineralised bone nodules was used with reproducible results.

**Major Compulsory Revisions**

1. The authors use RT-PCR to measure changes in levels of gene expression. The PCR parameters detailed in the Methods section indicate 35 cycles of amplification in each reaction. Under these conditions the PCR is not quantitative and cannot be described as ‘semi-quantitative’. The major concern here is that the house keeping gene #-actin, which is usually highly expressed, has reached saturation levels and therefore could not be used to normalise the levels of expression of the other target genes. The exponential phase of the PCR amplification for each of the probe sets should be determined and the experiments should be repeated with the appropriate, empirically determined number of cycles for each gene.

   **In accordance with the Reviewer, text in Methods section has been modified to read:** “After the optimal number of cycles determined for each primer set, PCR products were separated by electrophoresis on 2% (w/v) agarose gel and were visualized by ethidium bromide staining” (p11).

   Semi-quantitative measurements are adequate since they are largely used through the literature. However, we did not detail each procedure for each series of primers in the Methods section bearing in mind that this is state-of-the-art. In any case, to answer the Reviewer, we partly repeated the experiments under quantitative conditions and saw no difference (annexed figure that we do not include into the paper but is for the kind attention of the Reviewers).

**Discretionary Revisions**

1. The great majority of the work focuses on the development and evaluation of the experimental system while dasatinib effects are only shown in figures 7 and
8. As figure 8 presents a gene expression study it requires revision. The manuscript could be improved by the addition of a bone nodule assay, similar to the one presented in figure 2A, for the study of the effect of dasatinib on nodule formation.

**Now, dasatinib effect is documented by 5 figures (Figs.6-10) and we confirm the results of the former Fig.8 (now Fig.9). Quantification of matrix mineralization was described in Fig.8A; at that state of the study, a quantitative analysis is more relevant than “bone nodule assay”**.

2. The decrease in expression of RANKL is interpreted as a possible indirect mechanism of inhibition of osteoclastogenesis by dasatinib. However, if the RT-PCR results are correct, the decrease in RANKL only occurs in the absence of DAG, while in the presence of DAG there seems to be a slight increase in RANKL expression. Also, the levels of OPG should have been determined in this experiment, as the ratio between RANKL /OPG is a more accurate indicator of the effect of osteoblasts on osteoclastogenesis.

*This is now documented by presenting the effect of dasatinib on RANKL / OPG ratio in Fig.10.*

3. Table 2 presents the same data as figure 4 and could be omitted.

**Table 2 has been deleted.**

**Minor Essential Revision**

1 ‘Brc-Abl’ appears in a number of places and should be corrected to ‘Bcr-Abl’.

*This has been corrected.*

2 The last sentence of the introduction is unclear (...in which each associated stage was identified...).

*This sentence has been reformulated for better understanding.*

3 The number of passages used for the bone marrow derived cells should be added to the methods.

*Done in p7*

4 Methods, page 11, second line: ‘..upstream sense and downstream sense primers...’

*Replaced by “forward and reverse primers”?*

5 In the Results section, there is a reference to figure 2A then to 3A followed by 2B and 3B. The authors should consider changing the figures in a way that will enable their sequential mention in the text.

*Figures 2A and 2B were combined to require only one color figure.*

6 The following sentence appears in the Discussion (First paragraph, page 21)
‘Our data added to the previous studies strongly support that Src kinase activity is the main target for dasatinib in MSC differentiation process’. However, the experiments described in the manuscript do not address this question, and the only evidence for that is an experiment performed with another Src inhibitor that produced similar results. As this additional experiment is not part of the current manuscript there is no data here to show that the main target of dasatinib in MSC is Src.

It is known that dasatinib targets Src and that Src negatively regulates osteoblast differentiation. It is why we do not present some data (Src phosphorylation and effect of E804) in the first version of our manuscript (“data not shown”). BUT the Reviewer is right, it is not documented in MSC. Then, we added 2 figures (Fig.7 and Fig.8C) to support that Src inhibition by dasatinib stimulates MSC differentiation and we modified the text in p24 “Altogether, our data, added to previous studies from other groups [20] [22] [23] [27], strongly support that Src kinase activity is the main target for dasatinib in MSC differentiation process”.

Other points for consideration:

The manuscript adheres to the standards for reporting and the writing is acceptable. The abstract accurately describes the study, but the conclusions and the title are somewhat overstating the results. The only indications for stimulation of osteoblast differentiation by dasatinib are in figure 7; on day 14 calcium concentrations are higher with dasatinib and on day 7 ALP activity is higher. The gene expression data is not quantitative and therefore unreliable. The inhibition of RANKL occurs only in the absence of DAG on day 7 and the suggestion of an indirect inhibition of osteoclastogenesis is therefore based on a single experimental point.

Gene expression data (Fig.9) have been confirmed by real-time PCR (additional figure for Reviewers). We choose to use semi-quantitative PCR because we always have obtained relevant results with this technology as it is confirmed. However, as we have not the possibility to re-analyze the samples used in Fig.4 by real-time PCR and that we feel it will be “cumbersome” to present both RT-PCR and real-time PCR in “Methods” section for Fig.4 and Fig.9, respectively, we propose to keep RT-PCR results in the manuscript, and note that they were confirm by real-time PCR (p20). In addition, inhibition of RANKL has been also confirmed by ELISA and is now documented by 2 independent analyses (Figs.9&10). With these additional data, we think that our results fully support our conclusions.

Level of interest: An article whose findings are important to those with closely related research interests
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
Reviewer's report #2

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

Version: 1 Date: 4 November 2009

Reviewer: Susannah M O'Sullivan

Reviewer's report:

General Comments:
This a well presented and focused study on the effects of dasatinib to stimulate differentiation of human bone marrow-derived mesenchymal stromal cells (MSC) into osteoblasts, and inhibit expression of RANKL, thus potentially indirectly effecting osteoclast maturation and activation.

This study is interesting in light of the known effects of imatinib on osteoblast differentiation and osteoclast activation, and the effects of Src inhibition on osteoblast function in vivo and in vitro.

The authors have appropriately ensured the reliability of the differentiation assay, established appropriate gene and functional markers of osteoblast maturation, and the appropriate dose range of dasatinib in their assays. A number of different functional and gene markers have been used. The observations are robust and of significant scientific interest. However the authors have not addressed the possibility that the effects they have observed are due to inhibition of Abl or another tyrosine kinase. Furthermore, the limitations of the study are not clearly identified.

Our hypothesis was that these effects are mediated through the inhibition of Src. To further support our data, we added an experiment showing the inhibition of phosphorylation of Src by dasatinib (new Fig.7 and comment in p18) and that E804, a specific Src inhibitor, induced the same effects as dasatinib on ALP activity in MSC (new Fig.8C and comment in p20). These additional results strongly support a role for Src in dasatinib effects.

Specific Points:

Discretionary Revisions:
In the Background, the second sentence is somewhat cumbersome and could be revised. In the last sentence of this section, ‘osteogenic differentiation’ would be more appropriately termed ‘osteoblastic differentiation’.

These two sentences have been corrected.

Why have the authors chosen to use RT-PCR rather than real-time PCR (QT-PCR) in assessing relative gene expression?

We choose to use semi-quantitative PCR because we always have obtained relevant results with this technology. To confirm this, we have re-analyze the
new Fig.9 using real-time PCR (see additional figure for Reviewers) and we found the same results. Thus, as we have not the possibility to re-analyze the samples of Fig.4 by real-time PCR and that we feel it will be “cumbersome” to present both RT-PCR and real-time PCR in “Methods” section, we propose to keep RT-PCR results in the manuscript, and note that they were confirmed by real-time PCR (p20).

Minor Essential Revisions:
In the Abstract, Brc-Abl should read Bcr-Abl. The first sentence of Methods subsection does not make sense and should be revised.

These have been corrected.

The last paragraph in the Results subsection ‘determination of osteoblast-related gene expression during MSC differentiation’ largely reduplicates information in the third paragraph in this section. These two paragraphs should be amalgamated.

The two paragraphs have been combined (p15) and Table 2 has been deleted to avoid repetitive information with Fig.5.

In the Discussion paragraph 6, the authors comment that their data ‘strongly support an addition indication for dasatinib therapy in patients with bone loss’. This is overstating the data; however it would be reasonable to assess the effects of dasatinib in an in vivo model.

It has been corrected in p25 (“Our data suggest that patients with bone loss could benefit from dasatinib therapy”).

Major Compulsory Revisions:
In the Background, some discussion of the relative inhibition of Bcr-Abl vs Src, as well as the known effects on bone of Abl inhibition should be incorporated.

Are any other tyrosine kinases effected by dasatinib? What steps have been taken to ensure that the observed effects are (purely) due to Src inhibition? In the Discussion, some further justification for attributing the observed effects to Src should be included, particularly given the similar effects on osteoblast differentiation and RANKL expression to those seen with imatinib, which is a PDGF/Kit/Abl/c-Fms inhibitor. While the observed effects are robust, the attribution to Src-inhibition is largely based upon circumstantial evidence, and this limitation should be acknowledged. If the authors’ own work with another specific Src inhibitor is to be used as evidence that the effects are due to Src inhibition, these data should be presented.

Comments (from Li Nat Genet 2000) on Abl and bone have been added in Discussion section (p23). Additional data on the effect of dasatinib on Src phosphorylation (Fig.7) and on the effect of the E804 Src inhibitor on ALP activity (Fig.8C) are now presented to support the central role of Src inhibition
in the stimulation of MSC differentiation by dasatinib.

As acknowledged by the authors, the functional and gene expression results from the differentiation experiments are not completely consistent, in particular the difference between ALP activity and ALP gene expression. Some further discussion should be provided regarding the reason for the discrepancies, and the reasons for the authors selecting certain markers as being more reliable than others. Furthermore, some attempt should be made at explaining dasatinib’s varying effect on gene expression with or without ‘DAG’.

**ALP activity is probably more reliable to the mineralization process (p23).**

The reasons for the selection of some markers are now emphasized in Results section (pp17,18).

**DAG (osteogenic medium) is necessary to induce a complete osteoblastic differentiation.** Dasatinib speeds up the differentiation process but is not sufficient, e.g. to induce matrix mineralization. This is why, in certain situation depending of the time of incubation, that combination can induce over-stimulations (Figs.8&9). Dasatinib is more a stimulator than an inducer of osteoblastic differentiation. Our conclusion indicated that “it is able to significantly stimulate the osteogenic differentiation of MSC”.

**Level of interest:** An article whose findings are important to those with closely related research interests

**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 I have no competing interests.
Reviewer's report #3

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.

We disagree with the affirmation of the Reviewer.

Indeed, the stimulatory effects of dasatinib are clearly and significantly detected (on calcium deposition after 14 days, on ALP activity after 7 days and on RANKL expression level at days 3 and 7). We also observed significant change in BSP and OPN expression. As dasatinib potentiate the effects of DAG, the effects of dasatinib are observed at specific stages of the differentiation process and are of course “transient”.

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.

Undifferentiated cells means MSC not exposed to DAG (as seen in Figs.9&10). We clarified “undifferentiated MSC” by adding “no DAG induction” both in Discussion (p24) and Conclusions (p26) sections. The effect of dasatinib on RANKL is now confirmed by real-time PCR and ELISA.

Difference between DAG and Dasatinib+DAG conditions on RANKL expression at day 7 (new Fig.9) is not significant (as indicated in legend, this comparison has been examined).

-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.

We provided additional proof for the inhibition of Src phosphorylation by 10^{-8} M dasatinib in MSC (Fig.7). DAG does not affect this effect and E804 inhibits similarly Src phosphorylation (data not shown) (p19).

-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 effects of glucocorticoids should, in our opinion, be addressed separately in another at least one full paper.

-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.

We disagree again with the Reviewer: as replied above, our data do support a stimulatory effect of dasatinib on MCS into osteoblastic differentiation.

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

This has been addressed in the new Fig.6B.
- 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.

We agree with the Reviewer; This is why we had specifically examined the significance between DAG and DAG+dasatinib (as it was written in the legend of former Fig.7 “** p<0.05, ** p<0.01 and *** p<0.001 versus the corresponding control, and comparing DAG versus Dasatinib+DAG”). In Figs 8&9, we found significant differences between both conditions.

- 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).

Of course, this model and dexamethasone effect are known but specific markers for each stage of the differentiation process are not clearly defined through the literature. This has been addressed in the Background section (p5). In our study, we described a suitable combination of markers and used it to evaluate the effects of dasatinib. The manuscript as is revised now contains 5 out of 10 Figs dealing with this issue.

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.

Figs 3B and 7B could appear redundant and slightly different. This is due to the fact that MSC are from different donors in these 2 experiments. Consequently, “control conditions” are mandatory and not redundant with however slight differences in the results but with the same tendency.

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 Table 2, expression levels (0 to ++++) were scored independently of their significance. Moreover, Table 2 has been removed (information in Fig.5 instead). OSX was not further used as “marker” of osteoblastic differentiation.
because of its lower range of variation between day 0 and day 21.

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.

We do not agree with the Reviewer since we show in Fig.9 (former Fig.8) that BSP was significantly modulated by DAG at day 21. At days 7 and 14, difference between Fig.4C and Fig.9 can be explained by the use of different donors of MSC, requiring in both control and DAG conditions to be repeated. However, the same results (BSP mRNA level modulation in DAG condition) were obtained at day 21.

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.

As shown in Fig.4C, the change in expression of these genes (Runx2, type I collagen, OSX) did not reach significant levels as to be used as markers of osteoblastic differentiation and therefore they could not evaluated in dasatinib experiments. In addition, the activities/functions of such factors (especially transcription factors) are known to be not fully related to their level of expression so they cannot be reliably used as markers.

- 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?

DAG weakly reduced the growth of MSC without apparent sign of toxicity. This is documented in Fig.6B. On the other hand, MSC in differentiation phase are less proliferative.

- 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 optimal number of cycles was determined specifically for each primer set, as written now in Methods section (p11).
-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?

This is now better presented in legends of figures. Results represent mean of n experiments performed each in duplicate. Each experiment was performed with MSC form one single donor.

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

It is what we obtain !!

-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.

mRNA expression was NOT significantly different. This is clearly indicated since no star has been added.

-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?

As notified in Fig.9 (former Fig.8), it is (a star is added). This is confirmed by real-time PCR (additional figure for Reviewer).

-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.

It is corrected.

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

We thank the Reviewer for this comment, ALP activity (U, µmol p-nitrophenol released per min) was now normalized for 10^5 cells.

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

Unfortunately, no samples still available for normalization in this way. However, cell density is not significantly different among culture conditions (cells were cultured up to confluence before differentiation induction), so
The culture medium volume can be used as reference.

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?

Done in p7.

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

10⁻² M, now indicated in p8.

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

No difference has been observed.

- 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?

The stability of dasatinib in human plasma was assessed at room temperature up to 48 h. The variations over time of the drug was comprised within the ±15% of starting concentrations indicating that dasatinib can be considered stable at room temperature. (Haouala, Journal of Chromatography B, 877, 2009).

- Misspelling CBFA1 -> RUNX2 on figure 4B.

Corrected

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