Author's response to reviews

Title: Modelling NF1 tibial dysplasia and its treatment with lovastatin.

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Author's response to reviews: see over
Dear Editor,
We thank for reviewing our ms entitled “Modelling NF1 tibial dysplasia and its treatment with lovastatin.” ms# 1667189777189248. Please find below our answers to the reviewer’s comments.

Reviewer 1 (David D. Little)

1. Group sizes are a bit confusing - one can infer total mice = 24 and group size n=6 was used from the abstract and paragraph 2 of the results. Group sizes should be put in the methods. Seeing as decalcified and resin histology were done, were groups split n=3 and n=3 for histology? Were all n=6 used for uCT outcomes? What happened to the data from the 4th group (i.e. lovastatin treated controls)? A table or some delineation of numbers is required.

We have added the delineation of the mice quantities in the methods section of the Abstract:

“At each experimental time point 3 Lovastatin treated mutant mice, 3 untreated mutant mice and 3 untreated control mice were analysed. The animal group sacrificed on day 14-post injury was expanded to 6 treated and 6 untreated mutant mice as well as 6 control mice.”

24 mutant mice and 12 control mice were approved by the local ethics commission to be involved in the test. No lovastatin-treated control group was involved, since the ethics commission argued these experiments were done previously and were not critical to the experiment.

We originally planned to analyse the healing process at 4 time points up to day 42. However, since the injury regeneration was already complete at day 28 we decided to restrict the analysis to day 7, 14 and 28 and to enlarge the group analysed at day 14, hence n=6 on day 14.

Information on the number of animals used was also introduced in the Figure 3 legend and corrected in the results section:

“Quantification of the mineralized matrix in the cortical regions (red ROI) and bone marrow shaft (green ROI) 7 days (n=3) and 14 days (n=6) post injury.”

“Unmineralized bone was detectable in all tested animals on the 7th and 14th day post injury (n=9) but it was consistently absent in the non-injured Nf1Prx1 tibiae.”

Additionally, missing information about bilateral injury induction was added into methods section of the Abstract:
Cortical injury was induced bilaterally in the tuberositas tibiae in Nf1Prx1 mutant mice and littermate controls according to a previously described method (Campbell et al., 2003). Paraffin as well as methacrylate sections were analysed from each animal.

2. The Ras signalling work was done in calvarial lysates where the Prx-Cre should not be expressed. The Nf1 -/- cells should only be in the limbs. Thus the cells from calvaria should be Nf1+/+? This would mean there should be no differences in Erk. Marrow cells taken from the limbs or bones of the limbs would surely be better to look at this signalling?

The Prx1-Cre mediated recombination inactivates NF1 efficiently in the calvarial bones see Figure1A as well as Supplementary Figure 1 (Kolanczyk et al., 2007). Since the purpose of this experiment was to investigate the systemic effect of lovastatin in the bones, we opted for the analysis of the whole calvaria bone lysates. A cell isolation was not necessary, as the calvarial bone are relatively easily dissected from surrounding tissue. The lysate preparation procedure was introduced in the Methods section. In the introduction we added a statement about the specificity of Prx1Cre mediated knockout, stressing cranial inactivation as well as types of affected cells:

“In order to better understand Nf1 function in bone we recently generated mice bearing a homozygous Nf1 inactivation in the embryonic limb and in the cranial mesenchyme. The affected cell types include endothelial cells, chondrocytes and osteoblasts but not osteoclasts, which are of hematopoetic origin (Kolanczyk et al., 2007).”

**Minor Essential Revisions:**

1. **The results section is written in present tense rather than past tense.**

The form was changed into past tense.

2. **More methods detail should be given, particularly for preparation of lovastatin (still in brief), and calvarial lysate preparation (is this bone, bone cells…)**

Lovastatin activation protocol, calvaria bone lysate preparation and primer sequences as well as cDNA template source for amplification of the in-situ probes were added in the Methods section.

3. **Figures should be in the order referred to in the text (1,2,3… not 1,2,5…)**

The order of the figures was corrected to match the order of their appearance in the text.
4. Clarify that these are representative sections and of how many animals?

Clarification was added into each figure legend.

Discretionary Revisions:
It may be helpful to switch Fig 1 and Fig 2 around (show decalcified histology first).

Figure order was switched

We thank Dr. Little for reviewing the manuscript.

Reviewer 2 (Juha Peltonen)

General critique

- Some conclusions and introductory elements apart from those mentioned above are too speculative; these are exemplified below, and certainly recognized by the authors

The statement was corrected (see below).

- The methods and introduction need more details, even though properly referenced. A study has to be independently readable. Just for example, even the Nf1Prx1 mice need to be introduced in more detail in the text, and the sequence specificities of the probes for in situ hybridization lack necessary details.

The description of the Prx1-Cre mediated recombination specificity was added in the Background section:

“In order to better understand Nf1 function in bone we recently generated mice bearing a homozygous Nf1 inactivation in the embryonic limb and in the cranial mesenchyme. The affected cell types include endothelial cells, chondrocytes and osteoblasts but not osteoclasts, which are of hematopoetic origin (Kolanczyk et al., 2007).”

Primer sequences as well as cDNA template source for amplification of the in-situ probes were added in the Methods section.

Various expressions are not accurate, for instance: “RNA probes” on page 6 apparently refer to cRNA probes

The mistake was corrected

Abstract background (page2): Quote “Here we report results of experiments in which we used a cortical bone injury model to simulate Neurofibromatosis type I (NF1) associated bone changes, in particular pseudarthrosis”. This model system may have no direct relevance to NF1-associated osseous lesions, and the
statement goes beyond clinical rationale.

The statement was replaced by:

“We studied bone healing in a cortical bone injury model in Nf1Prx1 mice as a model for NF1 associated bone disease.”

The manuscript does not tell, or at least it is not easily detectable whether the mesenchymal progenitors in Nf1Prx1 mice were heterozygous or homozygous for Nf1 inactivation. Even if it is evident from the previous publications, this information must be given here.

Was addressed in the Background section:

“In order to better understand NF1 function in bone we recently generated mice bearing a homozygous NF1 inactivation in the embryonic limb and in the cranial mesenchyme.”

“During normal bone repair, fibroblasts from the surrounding connective tissue along with mesenchymal progenitor cells from the periosteum”… This statement is not solid; the origin of cells becoming osteoblasts in fracture healing has not been proven in detail.

The statement was replaced by:

“Bone injury repair is a complex process, which requires the concerted effort of numerous cell types. It is initiated by an inflammatory response, which stimulates fibroblasts from the surrounding connective tissue to proliferate and fill in the injury site with a provisional extracellular matrix. In parallel mesenchymal progenitor cells from the periost are recruited into injury site to become osteoblasts.”

The pharmacological background of lovastatin should be explained in more detail on pages 4-5. Even though quite correctly stated “Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, broadly used for reduction of serum cholesterol. As statins inhibit the initial enzyme of mevalonate pathway, they also reduce prenylation and farnesylation of signalling molecules, such as Ras and Ras- related proteins”, important information is missing on drugs in clinical for osteoporosis and interfering the same mevalonate pathway, but on a different point. These drugs, bisphoshonates, mainly interfere with osteoclast function, which is also relevant to bone dynamics.

The following was added in the Discussion:

“Additionally, results of in-vitro experiments indicate that statins might inhibit bone resorption by interfering with osteoclast function in a similar way as bisphosphonates (Fisher et al., 1999). Both drug groups inhibit the mevalonate pathway albeit at different levels, thus their mechanisms of action overlap. The clinical relevance of this remains unclear as several...”
independent studies were published presenting contradictory results on the fracture risk reduction assessment in lovastatin-treated patients (Jadhav and Jain, 2006).

The Discussion notes (data not shown) that the number of osteoclasts at the site of experimental injury in mutant animals was not increased. It is unclear how the osteoclast count was performed. Also the mere number of osteoclasts as such does not necessarily correlate with osteoclast function, a factor which may potentially interfere with the results. It should also be highlighted that osteoclasts in this experimental model may not be Nf1 deficient.

We thank reviewer for this important comment. We performed quantification of the TRAP positive cells lining the lamellar bone within the injury site. Our original statement referred to the total number of TRAP positive cells per callus, which appeared not to be increased. However, when these numbers are related to the bone volume (BV), an increased number of TRAP positive cells in mutant animals as compared to controls become apparent. The increase appears to be of the similar magnitude as the one observed in the chondro-ossous junction in Nf1Prx1 growth plates (Kolanczyk et al., 2007). Lovastatin treatment slightly reduces the TRAP positive cell numbers but does not bring them back to control levels. This was further corroborated by measurements of bone turnover marker deoxypyridinoline (D-PYD) in serum.

In the result section we added the following:

“Osteopontin is known to facilitate osteoclast mediated bone resorption (Asou et al., 2001; Yoshitake et al., 1999). We thus quantified TRAP positive bone lining cells within the injury site and determined the rate of bone turnover by measuring deoxypyridinoline (D-PYD) concentrations in serum. Osteoclast numbers were increased in the injury site in Nf1Prx1 mice when compared to controls and Lovastatin treatment did not significantly change this (data not shown). This was paralleled by an increased serum D-PYD in Nf1Prx1 animals which was only slightly reduced by lovastatin treatment (control 2.5±0.39 nMol/l; Nf1Prx1 3.95±0.71 nMol/l; Nf1Prx1 +lovastatin 3.49±0.83; n=3).”

In the method section we added the following:

“Serial, 6µm thick paraffin sections were prepared and used for Masson-Goldner staining, in-situ hybridisation and TRAP staining. TRAP histochemistry and TRAP-positive regions quantification was performed on the paraffin sections as previously described (Kolanczyk et al., 2007).”

“Serum Deoxypyridinoline (D-PYD) determination
Serum D-PYD was measured with METRA Serum PYD EIA Kit (Osteomedical GmbH) according to supplied protocol.”
We corrected the statement on the osteoclast numbers in the discussion:

“Additionally, our data show an increased number of osteoclasts at the injury site (data not shown) paralleled by an increased serum D-PYD concentration in the mutant animals. Both effects are only marginally reduced by lovastatin treatment. The increase of osteoclast number in the injury site is similar to our previous finding of the increased osteoclast number in the chondro-osseous junction (Kolanczyk et al., 2007). This effect is likely cell non-autonomous, as Nf1 is not inactivated in Nf1Prx1 osteoclasts.”

In our opinion injury-induced bone demineralization in the Nf1Prx1 mice, shows features of tumor induced osteomalacia, and thus is not directly caused by osteoclasts. This is in agreement with the multiple reports of osteomalacia being observed in association with NF1 (Konishi et al., 1991).

We thank Dr. Peltonen for reviewing the manuscript.

Reviewer 2 (John C. Carey)

We thank Dr. Carey for reviewing the manuscript.

REFERENCES:


We hope that reviewer questions, suggestions and comments have been answered and hope the ms is now acceptable for publication in BMC medicine.

Sincerely

Mateusz Kolanczyk