Bone Research Unit 2017

The emergence of the human recombinant transforming growth factor-β3 (hTGF-β3) as the novel bone inductive morphogen for skeletal tissue regeneration in non-human and human primates

The Bone Research Unit Theme 2017: Initiating the induction of bone formation in non-human and human primates
2017 Bone Research Unit & current research activities

BONE RESEARCH LABORATORY - SCHOOL OF ORAL HEALTH SCIENCES - FACULTY OF HEALTH SCIENCES UNIVERSITY OF THE WITWARSRAND, JOHANNESBURG

Ugo Ripamonti – MD, DDS, MDent, Spec. Maxillo-Facial Surg, PhD (Med) – Full Professor B1 NRF rating 2017-2021; h Factor: 40; citations > 4433
Department of Oral Medicine & Periodontology, School of Oral Health Sciences, University and Gauteng Department of Health
Director: Bone Research Laboratory, Department of Oral Medicine & Periodontology, School of Oral Health Sciences, Faculty of Health Sciences, University

August 14th 2017

Introductory notes, Director’s Report
The Bone Research Laboratory (BRL), a Research Unit of the URC/FRC in the Faculty of Health Sciences, University, has now administratively settled since September 2014 under the aegis of the Department of Oral Medicine & Periodontology, School of Oral Health Sciences, University. The Unit has gained scientific momentum as judged by its high impact factor publications and awarded research grants.

The current position of Director is occupied by Professor Ugo Ripamonti who is a Specialist in Oral Medicine and Periodontology. Professor Ripamonti is a full-time consultant in the department of Oral Medicine and Periodontology, where he is responsible for coordinating research and postgraduate supervision.
Resume’ and Research Highlights

The research of the BRL is primarily focused on tissue biology and regenerative medicine. Human life expectancy has increased steadily; more humans are hence living long enough to suffer age-related loss of function and crippling diseases and there is a need to improve the health of older people. The world, however, is unprepared for the inequalities that this knowledge is generating between the world’s rich and poor. The BRL is thus geared to provide South African research knowledge and mechanistic insights into regenerative medicine for the population of Africa and South Africa. The BRL of the University is working so as to avoid the inequalities that the accruing knowledge of regenerative medicine and tissue biology are now so dramatically evident between the world’s rich and poor. No matter how South Africa is affected by sub-Saharan Africa exceptional persistence of poverty, granting agencies in South Africa and thus the University of the Witwatersrand, Johannesburg, should continuously provide knowledge for the ultimate benefit of the people of South Africa injecting funds to overcome the disparities and inequalities of tissue biology amongst the people of Africa and of the world. This, as an additional mechanism to escape the ‘poverty trap’ with the associated unhealthy poverty of scientific thinking. The Unit is thus set to provide research data that can be translated in clinical contexts, so that South African expertise is applied to treat patients affected by skeletal deficiencies implementing a regenerative scenario of molecularly re-built tissue parts for the human body.

In context, the BRL and thus the University are the only scientific enterprises worldwide to have shown that different and novel osteogenic soluble molecular signals initiate the induction of bone formation and in primates only. The Director has filed US, EU and WO PTC patents with the help of the Technology Transfer’ Unit of the University. The University has been awarded not one but three patents, two US and one EU patent. In contexts of the biological activities of the hTGF-β3 isoform and with the absolute novelty of the biological concept of “tissue transfiguration in vivo” it is now imperative that the US and EU patents (see research output) be licensed so as to finally ripe economical rewards after the multifaceted eclectic research work of the Unit. Regenerative medicine and tissue engineering are still failing the predictable and successful translation in clinical contexts of the novel therapeutic strategies so brilliantly generated in pre-clinical animal models which, without enough data for translation, sparked the promise of engineering new tissues and organs for the human body. This promise later morphed into the hyperbole of promised novel regenerative treatments based on pre-clinical data mainly in vitro and in vivo models in rodents. Our constant interrogation of cellular differentiation and osteogenic potency in vivo through the addition of novel experimental systems in the non-human primate Papio ursinus has led to a mandatory re-evaluation of the mechanistic signals that frame our understanding of the induction of bone formation in primates’ species and possibly, the understanding of the fundamental biological mechanisms of unique human biology, as highlighted in two Leading Opinion Papers in Biomaterials (IF>9) Ripamonti et al. 2014; Ripamonti et al. 2016).
The dawn of this 2017 research year and the five-year cycle ahead of the Unit sees the Bone Research Laboratory and its Director in full transitional phase between the bone morphogenetic proteins (BMPs) – previously used at isoa by the BRL in pre-clinical and clinical contexts in the non-human primate Papio ursinus and in humans, televised worldwide by Beyond 2000 in 1996/97 - and the thoroughly novel use of the third mammalian recombinant human transforming growth factor-\(\beta_3\) (hTGF-\(\beta_3\)), strikingly endowed with the unique capacity to initiate rapid and substantial induction of bone formation but in primates only (Ripamonti et al. 2008 *J Cell Mol Med*; Klar et al. 2014; Ripamonti et al. 2014; Ripamonti et al. 2015; Ripamonti et al. 2016 *Biomaterials* x4).

Since then all of the strength of the Unit has been primarily focussed to assign the mechanistic insights of the induction of bone formation by the hTGF-\(\beta_3\) isoform combined with coral-derived macroporous constructs when implanted in the rectus abdominis muscle of Papio ursinus (Klar et al. 2014; Ripamonti et al. 2015, *Biomaterials* x2). The Unit did also provide the mechanistic insights into the limited induction of bone formation in calvarial defects shown to be antagonized by Smad-6 and -7 over-expression. In a full paper to *Biomaterials*, TGF-\(\beta\) signalling via inhibitors of DNA binding-2 and-3 (ID2,ID3) creates permissive or refractory microenvironments that regulate the induction of bone formation (Ripamonti U et al. 2016). Our innovative experiments in Papio ursinus have shown that the induction of bone as initiated by the hTGF-\(\beta_3\) isoform in the rectus abdominis muscle is via the bone morphogenetic proteins gene pathway with hTGF-\(\beta_3\) controlling the induction of bone formation by regulating the expression of BMPs via Noggin expression. This unequivocally demonstrates that hTGF-\(\beta_3\) elicits bone induction by up-regulating endogenous BMPs and it is thus blocked by Noggin. Our combined data using the mammalian hTGF-\(\beta\) isoforms indicate thus that the TGF-\(\beta\) proteins act upstream of the bone morphogenetic proteins, inducing heterotopic bone by expressing BMPs genes and related gene products (Ripamonti et al. 2014; Ripamonti et al. 2015, *Biomaterials* x2).
Figure legend:
(A) Florid induction of bone formation by 125 µg recombinant human transforming growth factor-β3 (hTGF-β3) (dark blue arrows) within the macroporous spaces of the coral-derived construct; (B and C) Macroporous constructs pre-loaded with binary application of 125 µg hTGF-β3 and 125 µg hNoggin: minimal if any induction of bone differentiation (a minor island only in B dark blue arrow) or complete lack of bone differentiation (light blue arrows C) throughout the macroporous spaces. Bone formation is initiated by the hTGF-β3 which set the induction of bone formation by expressing BMPs that induce de novo bone formation but are blocked by hNoggin (Ripamonti et al. 2014; Klar et al. 2014; Ripamonti et al. 2015 Biomaterials x3).

Importantly, the reported mechanistic insights on the induction of bone formation by the hTGF-β3 osteogenic device did additionally offer a temporospatial molecular background behind the limited and substandard induction of bone formation by recombinant hBMPs in clinical contexts. Physiological expression of endogenous BMPs genes and gene products by the direct implantation of hTGF-β3 with TGF-β3 and BMPs gene expression do physiologically escape the antagonistic expression of Noggin, whereas the direct implantation of far too high doses of exogenously applied recombinant hBMPs in clinical contexts sets into motion the over expression of Noggin ad other inhibitory byproducts tightly controlling the bone induction cascade in humans as shown by the limited effectiveness of recombinant hBMPs in clinical contexts.

Induction of periodontal tissue regeneration & molecular insights into cementogenesis
Concurrently to the seminal studies on the induction of bone formation by the hTGF-β3, the Unit has also significantly contributed to the induction of periodontal tissue regeneration by the osteogenic proteins of the TGF-β supergene family showing as a world-first the bona fide induction of Sharpey’s fibres inserted into newly formed cementum as initiated by hTGF-β3 in Matrigel® matrix in Class II furcation defects of Papio ursinus.
This culminated in preparation of a major review published by the J Periodont Research that re-defined periodontal tissue regeneration in primates and sets the new rules of periodontal tissue engineering (Ripamonti U. Re-defining the induction of periodontal tissue regeneration in primates by the osteogenic proteins of the transforming growth factor-β supergene family. J Periodont Res 2016; 51: 699-715).

The Unit has additionally provided the world’ first in vivo study correlating tissue induction and morphogenesis by the hTGF-β3 with a time gene expression’ study. We did show that cementogenesis and osteogenesis as initiated by the hTGF-β3 isoform in furcation defects of Papio ursinus entails the expression of TGF-β3, Osteocalcin with fine tuning and modulation of BMP-2 and OP-1, and up-regulation of Cemp1 (Cementum Protein-1), within the harvested cementum (Ripamonti U. et al. J Clin Periodontol 2017; 44: 83-95)(IF 3.95).

The hTGF-β3 Master gene and gene product: Transfiguration of neoplastic masses into bone: Tissue transfiguration in vivo
The robust induction of bone formation by hTGF-β3 in the non-human primate Papio ursinus has forced a re-evaluation of the mechanistic insights of the induction of bone formation in primates, including humans. The morphological and molecular evidence of the rapid transfiguration of muscle tissue into bone by the hTGF-β3 osteogenic device has indicated to the Unit a further novel and as yet totally unexplored biological function of hTGF-β3, that is, the injections of hTGF-β3 into malignant neoplastic masses to induce the rapid transfiguration of the injected masses into bone for easier surgical ablation and surgical debridement. The injections of high doses of the hTGF-β3 isoform into neoplastic masses would “ostoegenize” the tumour, transfiguring all available responding cells into osteoblastic-like cells, possibly altering not only the neoplastic phenotype, but also the neoplastic genotype thus controlling differentiation so as to osteogenize secondary masses as well (Ripamonti U. US 2012/0277879 A1 Nov. 1, 2012; US 9,084,757 B2 July 21st, 2015; Ripamonti U. EP 1948218 B1 April 15th, 2015; Ripamonti U. 2016 Rapid induction of bone formation by the hTGF-β3 isoform. 3.4 hTGF-β3 master gene and gene products: Transfiguration of neoplastic masses into bone. CRC Press, USA).
Harvested human oral squamous cell carcinoma (SCC) fragments are collected from ENT surgeons debriding human patients diagnosed with oral SCC (Human Ethics clearance by UR and the BRL). Fragments are then implanted subcutaneously in athymic scid mice. Developing tumour-like masses (top right image) are then injected with doses of hTGF-β3 in Matrigel matrix kept liquid on ice and later harvested for molecular and histological analyses (Bone Research Laboratory, unpublished data 2017).

List of publication and research output: International Journals, Patents, Books and Book Chapters for the Specialist


Edited Book for the Specialist:

*Induction of Bone Formation in Primates - The Transforming Growth Factor-β3*

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<thead>
<tr>
<th>Name of student</th>
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<tr>
<td>Carlo Ferretti</td>
<td>PhD</td>
<td>Male</td>
<td>Oral Health Sciences Bone Research Laboratory</td>
<td>Self</td>
<td>2017 in progress</td>
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<tr>
<td>Rolando Klar</td>
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<td>N Vafaei</td>
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<td>Oral Health Sciences/Maxillo Facial &amp; Oral Surg.</td>
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<td>Bursary from Libya</td>
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<td>Nicolas Tagliatti</td>
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<td>DoH</td>
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Beside the students above, Ruqayya Parak of the School of Oral Health Science and Senior Research Technologist at the Department of Oral Biology part-timely seconded to the Bone Research Laboratory did complete her 3rd workshop at Exakt Germany to further improve and hone her significant skills to cut and process undecalcified sections using the Exakt diamond cutting saw, the only operating Exakt diamond cutting saw in Africa. Ruqayya prematurely passed away June 2017 and a technology post has been recently motivated in occasion of the Bone Research Unit quinquennial review 2014-2017 and application for FRC/URC recognition 2018-2022.
Research Grants

2016 National Research Foundation for B1 rating: R 80,000.
NRF Blue Skies Research Award: Concept Notes 2015/2016: R 191,000
Wits Seed Funds TIA Grant 2015/2017: awarded R 370,966,39
2017 National Research Foundation for B1 re-rating: R 80,000.
NRF Concept Notes NRF Blue Skies application 2018 BS170405225816 Why Homo sapiens do not heal like a baboon? Submitted/Pending
NRF Research grant for B1 rated scientist 2018 CSRP 170407226216: Does cementum initiate the induction of bone formation? Submitted/Pending
Wellcome Trust Senior Investigator Award: under preparation

New research initiatives

Additional time studies of the induction of bone formation by the hTGF-β3 in non-human primates *Papio ursinus*;
Induction of mandibular regeneration by hTGF-β3 and molecular and morphological analyses on day 15 and 30 of osteointegrating geometrically-defined titanium surfaces implanted into hTGF-β3/mandibular regenerates

What next? Or perhaps more provocatively, what’s now beyond the corner? Our latest papers and particularly the *Leading Opinion Paper* in Biomaterials (IF >9) (Ripamonti et al. 2016) has once again directly alluded to the limited translational effectiveness of recombinant human bone morphogenetic proteins to predictable regenerate the bone/bone marrow organ in man. The greatest challenge ahead of the BRL is to now start to investigate the molecular and genetic profile of this variable regenerative capacity not only between genera but particularly between non-human and human primates. The collaboration with the laboratories of molecular biology headed by Raquel Duarte, PhD, of the Department of Internal Medicine, School of Clinical Medicine will be pivotal to finally initiate the molecular dissection of different regenerative traits amongst primate species, i.e. *Papio ursinus* vs. *Homo sapiens*.

Our current studies have proposed that the high concentration of the hTGF-β3 isoform may directly re-program differentiated cells invading the macroporous bioreactors into osteoblasts rapidly secreting bone matrix. This will be a seminal and as yet unreported functionality of the hTGF-β3 isoform; the 2012 Nobel Prize winners Shinia Yamanka and co-authors in two Cell papers only reported the de-differentiation to embryonic stem cells status of fully differentiated cells upon exposure to a series of factors to obtain de-differentiation, in particular Oct3/4, Sox2, c-Myc, and Klf4 (Yamanaka et al. in Cell 2006/2007). Whether the TGF-β3 isoform may directly induce stemness will be shown by our current experimentation of the time study of the recombinant hTGF-β3 isoform. Cell differentiation correlates with significant up-regulation of *RUNX*-2 and *Osteocalcin* expression and further gene markers will be evaluated to specifically study de-differentiation genes for stem cells status acquisition of stemness. The relatively high concentration of the doses tested of hTGF-β3 may reveal the governance of stem and progenitors cells. Such direct governance may be controlled by micro-environmental
selected cues in stem cell “niches” in perivascular and/or myoblastic compartments where the high doses of the hTGF-β3 directly re-program differentiated endothelial/perivascular and/or myoblastic cells into rapidly proliferating osteoblasts. Our current results have shown that the induction of bone formation is initiated by day 17 after heterotopic rectus abdominis implantation (unpublished data 2017). No bone formation was seen in the available specimens prepared on day 15; the 15 days specimens were however critical for our morphological understanding of the pivotal role of the extracellular matrix’ patterning to control and direct cell differentiation.

Morphological analyses of hTGF-β3/treated macroporous bioreactors harvested on day 15 have revealed the differentiation of fibrin/fibronectin rings expanding within the coral-derived macroporous spaces structurally organizing tissue patterning and morphogenesis in hTGF-β3/treated macroporous bioreactors. We have shown that extracellular matrix rings provide structural anchorage for hyper chromatic cells, interpreted as differentiating osteoblastic-like cells re-programmed by the hTGF-β3 isoform from invading myoblastic/pericytic differentiated cells (see digital images below).

15 days time course hTGF-β3/treaded macroporous bioreactor

Molecular events are at the very basis of tissue patterning and morphogenesis, and molecular analyses at early time points together with undecalcified tissue sectioning may shed further and unexpected insights into the induction of bone formation by coral-derived macroporous bioreactors pre-treated with high doses of the hTGF-β3 isoform.

The digital image on day 15 above shows the complexities of the extracellular matrix’ patterning that proposes a tri-dimensional finely interlaced network that ultimately arranges a treading scaffold to host single cells suspended to a pseudo-like “arbor vitae” of extracellular matrix giving continuous molecular cues to pleiotropic differentiating pathways. De-differentiating cells nested within the newly deposited substratum’ rings rapidly differentiate into osteoblastic-like cells starting to secrete newly synthesized extracellular matrix.
Further studies are now under way to dissect molecularly and morphologically a time study of the induction of bone formation by the hTGF-β₃ isoform. Our previous experiments have shown that bone has not yet formed by day 15 in hTGF-β₃-treated macroporous bioreactors but rapidly invades the macroporous spaces with hyper cellular osteoblastic activity, angiogenesis and matrix secretion tightly bound to the macroporous bioreactors by day 30. Experiments were thus planned to molecularly and morphologically evaluate tissue specimens harvested on days 15, 17, 19, 21, 23, 25, 27, 30 and 90 after heterotopic implantation.

Unique undecalcified sections of the planned time study are now becoming available often containing not one but two coral-derived macroporous bioreactors with its associated generated tissues, as shown below:

![Image of tissue cross-section](image_url)

**90 days time course hTGF-β₃/treated macroporous bioreactor**
Polished ground section cut at 27 µm by the Exakt diamond saw grinding/polishing equipment of the Bone Research Laboratory; image courtesy of Ruqayya Parak.

The digital image above highlights a novel pattern of tissue induction as initiated by the hTGF-β₃ isoform when combined to coral-derived macroporous constructs. hTGF-β₃/treated specimens were implanted in the *rectus abdominis* muscle of *Papio ursinus* at different time points. The above digital image represents a set of generated constructs harvested on day 90 after heterotopic implantation showing two macroporous bioreactors resting just above the peritoneal fascia and surrounded by the *rectus abdominis* muscle. Note the powerful inductive wave of the induction of bone formation primarily initiating within the peripheral areas surrounding the heterotopically implanted hTGF-β₃/coral-derived bioreactor. Bone forms exclusively at the periphery [see right image] and it is followed by a robust substantial wave of bone formation that initiates outside the implanted bioreactor but it is generated within the bioreactor, and that after enveloping the bioreactor powerfully extend to contact the untreated control coral-derived construct, touching the macroporous construct of the control untreated bioreactor, blending with the “*intrinsic*” induction of bone formation that forms within the central/internal macroporous spaces of the control specimens.

The superbly cut, polished and stained undecalcified section by Ruqayya visibly shows how the primary differentiating events in untreated bioreactors develop within the macroporous spaces after stem cells invasion and differentiation; on the other hand, in hTGF-β₃/treated bioreactors, the adjacent muscle lucidly shows the development of a temporo/spatial induction of bone formation at the periphery of the bioreactor only, with a powerful
inductive wave that initiates within the bioreactor by the exogenously applied hTGF-β3 that result in the initiation of a sequential chain of cellular induction rapidly recruiting pericytic perivascular myoblastic cells adjacent to the implanted bioreactors directly transformed into secreting osteoblasts. Figuratively thus, the powerful inductive wave of the exogenously applied hTGF-β3 isoform transforms all available responding cells resting at the periphery of the implanted macroporous constructs with the rapid induction of bone formation so much so that there are no more cells to transform into bone nor cells have had the chance as yet to migrate intro to macroporous spaces which show of course the lack of bone differentiation. All of the instructive signals and of the available responding cells have been “consumed” so to speak at the periphery only of the hTGF-β3/treated bioreactor.
Induction of mandibular regeneration by hTGF-β3 and molecular and morphological analyses on day 15 and 30 of osteointegrating geometrically-defined titanium surfaces implanted into hTGF-β3/mandibular regenerates

The time has now arrived to further study mandibular regeneration in *Papio ursinus* by implanting the higher dose the Unit has used for mandibular regeneration in clinical contexts, i.e. the 250 µg dose of the hTGF-β3 isoform. Regenerated mandibles are later implanted with planar or geometrically modified titanium surfaces to study the osteointegration of titanium dental implants inserted into the newly generated bone, and to shed further morphological and molecular insights into the critical role of surface nanotopographies and geometry on the induction of bone formation and osteointegration.

*restitutio ad integrum* of large mandibular full-thickness defects, 3 cm in length, prepared in edentulized mandibles of *Papio ursinus* and implanted with recombinant hTGF-β3 14 months after implantation of 250 µg hTGF-β3 per gram of human demineralized bone matrix. Regenerates are trephined and inserted with titanium dental implants with planar or geometrically modified surfaces with a series of concavities (see below). Titanium devices are also implanted in the *rectus abdominis* muscle to study the effect of surface geometry on gene expression: which are the set of genes that are expressed by geometrically designed surfaces as opposed to standard linear surfaces?
Research Translation in clinical context

(a b) Severe craniomandibulofacial neoplastic mass requiring the surgical ablation of the left hemimandible temporally reconstructed with a silicon block as space maintainer with a titanium plate (c). (d) Radiographic digital image of the reconstructed mandible 15 days after implantation of 250µg hTGF-β3 per gram of human demineralized bone marix as carrier. Rapid induction of tissue morphogenesis within the implanted matrix. The image, representing a radiographic digital image 15 days post-implantation, is highly suggestive of advanced mineralization and gross morphology of mandibular and ramus restoration. (e, f) Complete regeneration of the human mandibular defects 6 months after hTGF-β3 implantation also resulting in the de novo induction of the ablated coronoid process. (g) Radiographic image 4 years after implantation of 250µg hTGF-β3 per gram of demineralized bone marix as carrier. Restitutio ad integrum of the operated hemimandible.
Concluding remarks and research perspectives

The Unit’s mission is now primarily focused to examine at the cellular and molecular level the mechanistic events leading to de novo bone induction by the hTGF-β₃ isoform and to translate laboratory and pre-clinical research in non-human primates *Papio ursinus* into clinical contexts and into intellectual property products, i.e. patents, methods, products, scientific knowledge know how and publications.

Within the available though very limited manpower, the Unit is successfully delivering its mission with a series of seminal novel papers in high IF Journals including few Leading Opinion Papers in Biomaterials (IF>9) as well as patented inventions in the US and EU patent office’s together with a unique CRC Press Volume on the induction of bone formation by the hTGF-β isoforms in primates including humans. A research technologist/histo-technologist is now urgently needed to continue to provide undecalcified histology on the two Exakt diamond saws available to the Unit. A developmental post from Gauteng Health has been just now awarded to the Unit from the School of Oral Health Sciences though long time will be needed to develop the incumbent into a positive force within the Unit without requiring full time developmental supervision from the Unit’ Director.

The Unit together with the molecular biology laboratory of Dr Duarte, Department of Internal Medicine, needs now to start to identify systematically the molecular and cellular basis responsible for the significant differences in healing patterns amongst mammals, i.e. to analyse molecularly and genetically the mammalian wound healing trait controlling the extent of tissue regeneration. Genome’ analyses between *Homo sapiens* and *Papio ursinus* may help to mechanistically reveal the substantial differences in healing patterns between the species spiralling regenerative medicine and tissue engineering alike to different biological platforms.