Citation: IBMS BoneKEy **11**, Article number: 575 (2014) | doi:10.1038/bonekey.2014.70

www.nature.com/bonekey

NEWS

Conversations with pioneers in the bone field: Henry M Kronenberg

Neil A Andrews

Brookline, MA, USA.

IBMS BoneKEy **11,** Article number: 575 (2014) | doi:10.1038/bonekey.2014.70; published online 17 September 2014

Collaborative research with developmental biologists led to a breakthrough in the understanding of endochondral ossification

Editors' Note: *This is the fourth in a series of interviews with investigators who have made groundbreaking contributions to understanding endocrinology, bone health and bone disease. See previous interviews with T John Martin* (http://www.nature.com/bonekey/knowledgeenvironment/2013/130424/bonekey201373/full/bonekey201373.html)*, Stavros Manolagas* (http://www.nature.com/bonekey/knowledgeenvironment/2013/130904/bonekey2013139/full/bonekey2013139.html) *and Ed Brown (*http://www.nature.com/bonekey/knowledgeenvironment/2013/131211/bonekey2013211/full/bonekey2013211.html).

Henry M Kronenberg, MD, is Professor of Medicine at Harvard Medical School and Chief of the Endocrine Unit at Massachusetts General Hospital, Boston, US. Dr Kronenberg has made landmark contributions to the field of bone biology. In particular, he elucidated a negative feedback loop by which parathyroid hormone-related protein and Indian hedgehog control chondrocyte differentiation in the growth plate, providing the basis for the current understanding of the cellular and molecular regulation of endochondral ossification. Dr Kronenberg spoke recently with Neil Andrews to discuss these seminal early findings, the path his subsequent research took and the unanswered questions that keep him excited for the future of bone biology research. An edited version of their conversation appears below.

BoneKEy: What was your path to scientific research?

Henry Kronenberg: It wasn't until medical school when I realized that research was something I wanted to do—I hadn't even been a science major in college. But when I was in medical school in the 1960s, it was a tremendously exciting time. Gene regulation in *E. coli* had just begun to be understood, and this was only a few years after the double helix was discovered. There was a symposium at Columbia, where I was a medical student, which brought together all the luminaries in molecular biology at the time: James Watson, Francis Crick, Sydney Brenner, Jacques Monod, François Jacob and Seymour Benzer. It was amazing and very inspiring, and I said to myself, this is the secret of life and what I've got to do. I did a little bit of research in medical school, and then after residency training I went to NIH [US National Institutes of Health] for two years and spent time in a lab that was studying gene regulation—this was just at the time that recombinant DNA was discovered.

BoneKEy: What did you decide to do after your time at NIH?

Henry Kronenberg: I was thinking about what I wanted to do next with my life, and an endocrinology fellowship made sense because, at the time, endocrinology was one of the few areas of internal medicine (which I had already been trained in) that was interested in gene regulation. I got an endocrine fellowship at Massachusetts General Hospital [MGH] where I had done my house officer training, and looked for a laboratory in which I could study gene regulation and even potentially do recombinant DNA work. There was only one group that was seriously interested in gene regulation at the time, and that was John Potts' group, which was studying parathyroid hormone [PTH].

My interest in PTH was not so much that I thought that calcium, bone, kidney and so forth were endlessly more fascinating than anything else. Rather, it was an opportunity that came along to study gene regulation. In fact, at this time in the early '70s, there were only a couple places in the world where serious recombinant DNA research was going on. One of them was in California—mostly in San Francisco—and the other one was in Cambridge—at Harvard and MIT. In fact, this was a time when recombinant DNA research wasn't even allowed at MGH because of concern about immunosuppressed patients and all the strange organisms that we were generating. So I went over to MIT into the lab of Alex Rich, who is a brilliant molecular biologist. He had a prior collaboration with John Potts to study the messenger RNA for PTH, and I went to Alex's lab to study the molecular biology of PTH; it became clear that this required the cloning of PTH cDNA as the next step. After spending a number of years in Alex's lab, I came back to MGH and set up a lab.

For the next decade, though, I didn't really think of myself as a bone person, but as a PTH/calcium physiology person. Our group was interested in PTH action—several different groups simultaneously were trying to clone the PTH receptor—and our group succeeded in doing so.[1]

BoneKEy: How did you become a bone biologist?

Henry Kronenberg: We started working on a number of models, in kidney and bone, to take advantage of having the molecular tool of the PTH receptor. One of the obvious things to do was to make use of gene knockout technology, which was the next new thing to come along. But we didn't know how to knock out genes, and so we had a crucial collaboration with Richard Mulligan at MIT, who had become quite competent at it. Andy Karaplis, my post-doc, went over to Richard's lab to learn how to work with embryonic stem cells, knock genes out, and do the initial insertion of a genetically engineered embryonic stem cell into a blastocyst to make a chimeric mouse. Andy brought PTHrP [parathyroid hormone-related protein] knockout mice back to MGH and we evaluated their phenotype.[2] Eventually we figured out that these mice had abnormal skeletons, and that's how I became a bone biologist. It turned out that PTHrP and the PTH receptor were important not just for bone development but also for adult bone biology, and so my interest in bone biology grew further.

BoneKEy: What was the state of knowledge at the time about endochondral ossification?

Henry Kronenberg: Most of the studies were limited because the endochondral ossification process was very difficult to recapitulate in cell culture and in organ culture. In retrospect, it turned out that when chondrocytes are taken out of their *in vivo* environment, they behave differently because they interact with matrix and adjacent cells differently. In addition, it turns out, as we found, that the perichondrial cells around the cartilage both signal to and receive signals from the cartilage that are crucial for understanding endochondral ossification, and that was not very well understood.

BoneKEy: What made possible the advances in the understanding of endochondral ossification?

Henry Kronenberg: Around the time that we knocked out the PTHrP gene, developmental biologists were using the tools of developmental biology, which at the time involved genetic manipulation of mice, and chickens, to understand more generally how all tissue is formed, and then bone became a special case.

In fact, a number of developmental biologists were lured into the bone field for two interacting reasons. First, the morphology of bone has everything to do with its function—in bone, structure is part of how the tissue works. Consequently, it turned out that when various signaling systems were manipulated genetically, that led to easily scoreable bone morphological phenotypes—changes in the morphology of bone that had profound implications for the function of bone both while bone was developing and after it developed. Thus a number of people began to study bone almost by accident; they were interested in other things, but what they ended up with was a skeletal phenotype that was easy to notice and define. Second, compared to the brain or the kidney, for instance, bone is a relatively straightforward tissue and organ in the way it is organized, so that certain questions were probably easier to answer in bone development than they were in other areas, and that was also appealing to people who were outside the bone field.

In addition, people don't need bones until they are born—without bones you can't get around, you can't breathe without a rib cage, and you die at birth if your bones are defective. But dying at birth is actually a very late time in the world of development, and in fact, there is a whole group of developmental biologists who look at what happens after birth as trivial and uninteresting extrapolations of what happens during development. Of course development does continue after birth and there are some interesting things to study, but for those who are interested in development, the idea that you didn't need bones until birth meant that you could have extreme phenotypes that didn't immediately destroy the organism. Letting the phenotypes play themselves out was a very attractive thing about endochondral bone development and led to a gigantic burst of activity in the field. My group was part of that, and what we brought to the study of endochondral ossification, just like those interested in developmental signaling pathways, was the ability to manipulate genes *in vivo* and look at *in vivo* phenotype readouts.

BoneKEy: How did you begin to work out the negative feedback loop by which Indian hedgehog and PTHrP regulate endochondral ossification?

Henry Kronenberg: There was a skeletal biology meeting in Washington, DC, and at that meeting I presented the PTHrP knockout work of Andy Karaplis from our group, which showed that in the absence of PTHrP, which was made at the top of the growth plate, chondrocytes differentiated into hypertrophic chondrocytes in an accelerated fashion earlier than they otherwise would have. We concluded that PTHrP's job was to slow down the differentiation of chondrocytes, and that's what I said at the meeting.

Then Cliff Tabin, who I would call a real card-carrying developmental biologist, gave a very different talk. He and his colleagues were isolating vertebrate versions of the hedgehog genes. In fact, what developmental biologists were doing at this time was taking all of the genes that Christiane Nüsslein-Volhard had shown were important for patterning in Drosophila—this is work for which she won the Nobel Prize—and realizing that these genes were not only important for setting up the developmental pattern in the fruit fly, but also that there were vertebrate versions of virtually all of these genes that turned out to be important for the developmental patterning of vertebrates and mammals. What Tabin and colleagues did was to isolate

vertebrate hedgehog genes, including Sonic hedgehog, Indian hedgehog, and Desert hedgehog, and then they started studying what each one of those genes did.

At the time, the style of discovery that Cliff had found most profitable was to use retroviruses to overexpress genes in the chick limb. Limb development was one of the classical developmental biology paradigms to which he had already contributed and that many people had been working on for decades before him. What he presented at the meeting was the consequence of overexpressing Indian hedgehog in the developing chick limb bud using retroviral infection. He found that high levels of Indian hedgehog expression stopped chondrocytes from differentiating into hypertrophic chondrocytes.

After his talk, I pointed out to him that his phenotype was in some respects the opposite of the phenotype of the PTHrP knockout, and that it was possible, therefore, that the two had something to do with each other. It was equally possible that there were many different pathways that were all important for making hypertrophic chondrocytes and that these were two interesting but unrelated pathways. But the Indian hedgehog phenotype certainly looked an awful lot like the opposite of the PTHrP knockout phenotype and made it possible that PTHrP regulated Indian hedgehog or that Indian hedgehog regulated PTHrP, or in some way or other they talked to each other in a common pathway for growth plate development.

Cliff expressed interest in following up that idea. I sent him some PTHrP probe, and Andrea Vortkamp from his group took the bones of chickens in which Indian hedgehog had been massively overexpressed and she did an *in situ* hybridization assay for PTHrP. What she found was startling: there was an enormous upregulation of PTHrP expression.

BoneKEy: What happened next?

Henry Kronenberg: The next crucial series of experiments was to take the mouse Indian hedgehog synthetic peptide, and add it to bone explants. Just as Cliff had found in the chick, when hedgehog protein was added to bone explants *in vitro*, it suppressed chondrocyte hypertrophy, but it didn't do so in bone explants from the PTHrP knockouts and it didn't do so with bone explants from the PTH receptor knockouts. That allowed us to argue that the synthesis of PTHrP was regulated by Indian hedgehog, and that PTHrP then slowed down the differentiation of chondrocytes, and that was required for Indian hedgehog's ability to slow down the differentiation of chondrocytes.[3]

That discovery also allowed us to put together the story of the PTH/PTHrP receptor knockout mouse; this is a mouse that Beate Lanske had made a couple of years earlier, but we hadn't published that work yet. The ability to integrate the PTH receptor into the PTHrP/Indian hedgehog feedback loop became a perfect way to present the PTH receptor knockout for the first time.[4]

BoneKEy: How did you learn more about Indian hedgehog's role in endochondral ossification?

Henry Kronenberg: Our early work provided an incomplete picture of the Indian hedgehog story. The next important part of the story came once again came at a meeting, this time at a Keystone conference on bone development that John Potts, Stephen Krane and I organized. Andy McMahon had knocked out the Indian hedgehog gene and Benoit St-Jacques presented that data at the meeting, and it was very exciting. Benoit presented all the things that Cliff and I hadn't noticed about Indian hedgehog action in bone from the overexpression studies. The knockout brought out several important lessons, one of which was that Indian hedgehog was absolutely required for the synthesis of PTHrP, and that in the absence of Indian hedgehog, there was accelerated chondrocyte hypertrophy—the phenotype was like the PTHrP knockout phenotype. I was delighted and felt that the Indian hedgehog knockout confirmed very rigorously, using completely different independent methodology, what Cliff and I had shown with overexpression data.

They also showed that Indian hedgehog, independent of PTHrP, was very important for direct actions on chondrocytes. They also found that in the absence of Indian hedgehog in the limb there were no osteoblasts. Indian hedgehog is absolutely required in the limb—and also in the vertebrae but not in the skull—for making osteoblasts, and a knockout experiment was needed to show that. Thus the Indian hedgehog knockout tremendously expanded the idea of what Indian hedgehog's role was in endochondral bone development.

What came out of this work was not only a beautiful paper from Andy McMahon's laboratory on the Indian hedgehog knockout,[5] but a collaboration, to this day, between Cliff's group, Andy's group, and my group. That two- or three-year period was a tremendously influential time in my own personal growth as a scientist because I have learned an enormous amount from the privilege of being able to collaborate with true developmental biologists. They're terrific.

BoneKEy: How did your work on endochondral ossification evolve after these early discoveries?

Henry Kronenberg: My research took several directions. One concerns the mechanism whereby PTHrP slows the differentiation of chondrocytes—all the work I've discussed so far doesn't speak to the mechanism. In fact, at the time, if we had wanted to discover the mechanism, it would have been impossible because there were several other key discoveries that were first required. What was needed was a molecular understanding of the regulation of chondrocyte hypertrophy. A complete understanding still doesn't exist, but we know a lot more now than we did then.

The first crucial discovery, by Gerard Karsenty[6] and Toshihisa Komori,[7] was that the Runx proteins were important not only for making osteoblasts, but also for making hypertrophic chondrocytes. The second crucial discovery, by the developmental biologist, Eric Olson, was that the transcription factor myocyte enhancer factor 2 (MEF2) in some ways is the master regulator of chondrocyte hypertrophy;[8] in the absence of MEF2C and MEF2D, there is no Runx2 [Runt-related transcription factor 2] in the growth plate and MEF2C has other actions on chondrocyte genes to drive hypertrophy. His hypothesis, which I think is correct, is that MEF2C and MEF2D are required for Runx2 expression. Eric Olson also showed that histone deacetylase 4 (HDAC4) is an important regulator of MEF2C in the growth plate.

Another very important contribution came from Andrew Lassar, who used primary chondrocytes and a chondrocyte cell line to argue that the way PTHrP works to stop chondrocyte hypertrophy is by driving HDAC4 out of the nucleus to allow MEF2C and Runx2 to drive the hypertrophic program.[9] This led to a collaboration between his group and mine to show that HDAC4 is a target of PTHrP *in vivo*; that work has been presented at ASBMR meetings and I hope it will be published soon. Shigeki Nishimori in my group has also shown that HDAC4 and HDAC5 work together to mediate the actions of PTHrP in

suppressing the hypertrophic program, and we've shown that through a series of overexpression and knockout experiments *in vivo*.

BoneKEy: What other paths did your research take?

Henry Kronenberg: Another direction concerns the actions of the PTH receptor in osteocytes and in osteoblasts. This involves projects that are separate from the work on endochondral ossification, and stem from my initial interest in PTH and the PTH receptor. They explore how PTH and PTH receptors regulate hematopoiesis. That's one area that has been a logical extension of the kinds of experiments that our knockouts make possible.

In more recent experiments we have been very interested in how we can use gene knockout and overexpression technology to understand the anabolic actions of PTH. The idea is to extend the tools of developmental biology into adult bones now that we can knock out genes out after birth and study phenotypes of adults and growing mice.

BoneKEy: What are the three or four burning questions for which you most want to know the answer?

Henry Kronenberg: One of them is the mechanisms by which PTH can dramatically increase bone mass—this is both a theoretically fascinating question as well as a phenomenally important translational question with regard to the treatment of osteoporosis. It seems likely to me that the answers will involve pathways, genes and processes that go beyond PTH and potentially could lead to new avenues of drug discovery and understanding of bone biology. We are studying this in a number of different ways.

A second question our group is very interested in, because I think it's an unanswered question in the field, is to delineate with more precision the early cells in the osteoblast lineage—the stem cells that have the potential to become osteoblasts, the osteoprogenitors that may be more restricted to the osteoblast lineage but are still very early cells, and then preosteoblasts that have started to differentiate into osteoblasts but are not yet osteoblasts. People in the field talk about those cells as if they know where they are, what they do, and how they are regulated, but in fact nobody knows. They may well be right and have genes and cells that fulfill various criteria that make cells plausible stem cells and osteoprogenitors, but it's very hard to prove that a specific progenitor cell normally becomes an osteoblast *in vivo*—it's a big unknown in the field. What we need to do is be able to point to stem cells, to osteoprogenitors, and to preosteoblasts, and find out what PTH and Wnt proteins do to each of those cell types *in vivo* in real-time during development and during remodeling. This has never been done and it is not known how to do it. We need to identify those cells and find the tools for studying them—it's a huge program, and many people are pursuing it.

This is very important work because we have some big mysteries on our hands. One is that we have a spectacular antibody against sclerostin that builds bone more than anything else does in our pharmaceutical armamentarium, and it does so for about six to nine months and then stops—and nobody knows why. PTH, to a lesser extent, does a similar thing: it builds bone for nine months up to a year or so and then stops—and nobody knows why. There may be many different reasons to explain these phenomena, but part of the answer could have to do with inadequate manipulation of all of the osteoblast precursors to make sure they keep coming. If you can't identify osteoblast precursors and know how they are regulated, then how can you answer the question as to why they don't keep coming?

BoneKEy: Thanks so much for speaking with BoneKEy about endochondral ossification and beyond.

Henry Kronenberg: Thank you for the opportunity.

Conflict of Interest

The author declares no conflict of interest.

References

1. Kronenberg HM, McDevitt BE, Majzoub JA, Nathans J, Sharp PA, Potts JT *et al.* Cloning and nucleotide sequence of DNA coding for bovine preproparathyroid hormone. *Proc Natl Acad Sci USA* 1979;**76**:4981–4985.
2. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM *et al.* Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev* 1994;**8**:277–289.
3. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 1996;**273**: 613–622.
4. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A *et al.* PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 1996;**273**:663–666.
5. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999;**13**:2072–2086.
6. Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev* 2001;**15**: 467–481.
7. Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M *et al.* Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev Dyn* 1999;**214**:279–290.
8. Arnold MA, Kim Y, Czubryt MP, Phan D, McAnally J, Qi X *et al.* MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev Cell* 2007;**12**:377–389.
9. Kozhemyakina E, Cohen T, Yao TP, Lassar AB. Parathyroid hormone-related peptide represses chondrocyte hypertrophy through a protein phosphatase 2A/histone deacetylase 4/MEF2 pathway. *Mol Cell Biol* 2009;**29**:5751–5762.