RESEARCH ARTICLE

Reduced β-catenin expression affects patterning of bone primordia, but not bone maturation

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ABSTRACT

Wnt/β-catenin signaling is involved in patterning of bone primordia, but also plays an important role in the differentiation of chondrocytes and osteoblasts. During these processes the level of β-catenin must be tightly regulated. Excess β-catenin leads to conditions with increased bone mass, whereas loss of β-catenin is associated with osteoporosis or, in extreme cases, the absence of limbs. In this study, we examined skeletogenesis in mice, which retain only 25% of β-catenin. These embryos showed severe morphological abnormalities of which the lack of hindlimbs and misshaped front paws were the most striking. Surprisingly however, calcification of bone primordia occurred normally. Moreover, the Wnt-dependent regulatory network of transcription factors driving the differentiation of cartilage and bone, as well as the expression of extracellular matrix components, were preserved. These findings show that 25% β-catenin is insufficient for the correct patterning of bone primordia, but sufficient for their mineralization. Our approach helps to identify bone morphogenetic processes that can proceed normally even at low β-catenin levels, in contrast to those that require high β-catenin dosages. This information could be exploited to improve the treatment of bone diseases by fine-tuning the individual β-catenin dosage requirements.

KEY WORDS: β-catenin dosage, Canonical Wnt signaling, Bone development, Limb patterning

INTRODUCTION

Canonical Wnt signaling activates the transcription of β-catenin-dependent target genes during many stages of bone development and maintenance (Duan and Bonewald, 2016), whereby a cytoplasmic multi-protein complex composed of the scaffolding proteins Axin and APC (Heuberger and Birchmeier, 2010; Munemitsu et al., 1995), and the kinases CK1α and GSK3β (Behrens et al., 1998; Munemitsu et al., 1995), tightly regulates the level of β-catenin. In the absence of canonical Wnt signaling this degradation machinery continuously phosphorylates β-catenin at its N-terminal region. This marks the protein for ubiquitination and proteasomal degradation (Aberle et al., 1997) and the transcription of canonical Wnt target genes is repressed. Upon binding of a canonical Wnt ligand to the co-receptor pair Frizzled and Lrp5/6 (Heuberger and Birchmeier, 2010), the degradation machinery is disassembled and β-catenin accumulates in the cell and is subsequently translocated to the nucleus, where it transactivates canonical Wnt target genes (Cadigan and Peifer, 2009; Heuberger and Birchmeier, 2010; Roose et al., 1998). Additional players that affect the level of β-catenin during bone development and repair include the extracellular Wnt inhibitors Dickkopf and Sclerostin (Florio et al., 2016; Ke et al., 2012; Qiang et al., 2008).

Early during embryonic development Wnt/β-catenin signaling plays crucial roles in determining the future body axes, and maintaining gastrulation and mesoderm production (Hikasa and Sokol, 2013; Holstein, 2012). During later stages, Wnt signals are important for induction, growth and organization of the future limbs (Kengaku et al., 1998). Hereby, reciprocal signals between mesenchymal and ectodermal cells promote the formation of the limb organizer, called the apical ectodermal ridge (AER) (Barrow et al., 2003; McQueeny et al., 2002; Narita et al., 2007). The AER promotes the proliferation of the underlying mesenchymal cells via Fgft signaling and induces the zone of polarizing activity (ZPA) at the posterior side of the growing limb bud. Limb patterning involves multiple signaling protein gradients (Wnt, Fgf and Shh), originating from either AER or ZPA, that in turn affect the expression of different Hox genes (Wellik and Capek, 2003; Capdevila and Izsósi Belmonte, 2001; Chiang et al., 2001). The individual bones of stylopodium (humerus or femur), zeugopodium (radius/ulna or tibia/fibula) and autopodium (hand or foot) are initially set up as aggregates of tightly packed mesenchymal cells (Hall and Miyake, 2000). These mesenchymal condensations differentiate into cartilage and are later replaced by bone via endochondral osteogenesis. The orderly progression of these processes again crucially depends on the timely activation of Wnt/β-catenin signaling in the respective cell types (Lu et al., 2013; Tamamura et al., 2005). The molecular mechanisms underlying bone formation are highly complex and involve the activation of mutually exclusive transcription factors and extracellular matrix genes in chondrocytes and osteoblasts. As the master regulator of chondrogenesis, Sox9 drives not only the expression of cartilage-specific collagen type II and Aggrecan, but also maintains the proliferation of chondrocytes and inhibits their differentiation into osteoblasts and thus ossification (Lefebvre and de Crombrugghe, 1998; Dy et al., 2012; Hattori et al., 2010). The activity of Sox9 is itself regulated by several upstream signals, of which canonical Wnt signaling exerts an inhibiting effect. Lastly, Sox9 inhibits the expression of Runx2, a key transcription factor for bone development (Liu and Lee, 2013). Conversely, activation of canonical Wnt signaling in pre-osteoblasts triggers the expression of Runx2 and other bone-promoting factors such as Sp7 or Atf4 (Huang et al., 2007; Yang and Karsenty, 2004). Their importance for osteoblast differentiation has been shown in knock-out mice or overexpression studies, which result in loss of bone formation or ectopic ossification, respectively (Komori et al., 1997; Ueta et al., 2001). Furthermore, these transcription factors activate genes like type I collagen alpha 1, osteocalcin or bone...
sialoprotein that are characteristic for the extracellular matrix of bone (Ducy et al., 1997; Kern et al., 2001; Ganss et al., 1999).

It is therefore not surprising that many human genetic diseases are associated with mutations that cause a loss of function (LOF) or a gain of function (GOF) of different genes of the Wnt signaling pathway. GOF mutations in both mice and humans lead to unusually high bone mass (Regard et al., 2012), while LOF mutations are usually associated with low bone mass. One of the most extreme LOF mutations is the Tetra-amelia syndrome. It is caused by mutations in WNT3, which leads to the complete loss of the formation of all four limbs (Niemann et al., 2004). In this study, we restricted β-catenin expression during bone development without disturbing the machinery of the Wnt signaling pathway. To this end, we conditionally expressed β-catenin only from the ROSA26 locus (Rudloff and Kemler, 2012) in all tissues caudal to the heart (Hierholzer and Kemler, 2009). We found that the morphogenesis of the distal forelimbs, hindlimbs and the tail was disturbed. We further showed that the expression of genes underlying the patterning process in the forelimb autopodia was severely disturbed. Yet, the formation of mineralized matrix in these bone anlagen appeared to proceed normally. More surprisingly, we could show that the underlying molecular machinery that controls the differentiation of chondrocytes and osteoblasts was preserved in β-catenin knockdown mice. Thus, low β-catenin expression levels are insufficient for correct patterning of the skeleton, but adequate for the maturation of skeletal primordia.

RESULTS
Knockdown of β-catenin leads to developmental defects in the caudal embryo

Expression of Cdx1::Cre mediates recombination in the three germ layers of the primitive streak region throughout the posterior embryo, caudal to the heart at mid-gastrulation (Hierholzer and Kemler, 2009). Thus, in (Cdx1::Cre)(β-catenin flox/flox) (ROSA26::β-catenin) mice, the endogenous β-catenin alleles are deleted and replaced by the ROSA26::β-catenin transgene. Homozygous expression of ROSA26::β-catenin leads to a β-catenin expression level of 25% compared to wild-type mice (Rudloff and Kemler, 2012). These mice, which exhibit severe malformations, are referred to as knockdown or mutant embryos throughout the text. For a better understanding of where the Cdx1::Cre mediated manipulation is active in our mutant mice, we performed histological and genetic analyses. Sagittal sections of embryonic day (E) 18.5 knockdown embryos revealed that internal organs (e.g. heart, intestine, kidneys, liver, lungs and pancreas) were present, excluding a general defect during early gastrulation (Fig. S1). Furthermore, we isolated DNA from these organs, as well as from brain, skin (head and abdomen), abdominal muscles and tail. Subsequent genotyping for endogenous β-catenin revealed that all tissues of foregut endodermal origin (i.e. small intestine, liver, lung and pancreas), anterior mesoderm-derived structures such as heart and spleen, and ectodermal organs like brain and skin from the head region still carried a floxed β-catenin allele (324 bp band in Fig. S1). This means that Cre recombination had not taken place in these tissues. On the other hand, all posterior mesoderm-derived tissues (e.g. kidney, abdominal muscles and tail) and skin isolated from the caudal half of the embryo lacked the floxed allele and only showed the deleted β-catenin gene product (500 bp band in Fig. S1). Taken together, Cre-mediated recombination in our knockdown embryos took place in all mesodermal and ectodermal tissues that are derived from the posterior embryo at midgastrulation, including the fore- and hindlimb primordia.

Phenotypically, mutant embryos showed major developmental defects with increasing severity in the caudal half of the embryo (hindlimbs and tail), as well as in the distal parts of the forelimbs (Fig. 1; Fig. S2). In more detail, most mutant embryos did not have any hindlimb structures (Fig. 1A,C and G), displayed a spina bifida (white arrowhead in Fig. 1A,B), an unusually curled tail (asterisk in Fig. 1A,C and Fig. S2A) and only rudimentary pelvic bones (black arrowhead in Fig. 1C). In very few embryos, truncated hindlimbs were present (black arrowhead in Fig. S2A). Forelimbs were always developed; however, their appearance was abnormal, and the digits showed severe malformations (Fig. 1A). To gain more insight into the skeletal changes, we performed Alcian Blue (cartilage) and Alizarin Red (calcified bone) staining on whole mount embryos (Fig. 1C,D; Fig. S2A) and isolated forelimbs (Fig. 1E,F; Fig. S2B-D). Shape and size of the skeletal elements of forelimb zeugopodia and stylopodia seemed not to be altered between control (Fig. 1F) and β-catenin knockdown embryos.
(Fig. 1E; Fig. S2B-D). In rare cases, the forelimb zeugopodium consisted of only one bone (Fig. S2B). Conversely, the autopodia of β-catenin knockdown embryos always showed severe morphological alterations, with carpals, metacarpals and phalanges exhibiting irregular proportions and orientations (Fig. 1E; Fig. S2B-D). Hereby, the number of digits could be either increased (Fig. 1E) or reduced (Fig. S2B,C) and in some cases fused phalanges were detected (Fig. S2D). Micro CT scans of control and mutant embryos (Fig. 1G,H; Fig. S2E,F) confirmed the findings of the skeletal staining. Moreover, in knockdown embryos, broadened, split lumbar vertebrae were found (white arrowhead in Fig. 1G), providing a morphological explanation for the spina bifida. Furthermore, in mutant embryos the ribs protruded at a flatter angle compared to controls and the intercostal distance seemed to be increased (Fig. 1G,H; Fig. S2E,F). Interestingly, there were only 11 instead of the expected 12 rib pairs present in the micro CT-scanned knockdown embryo (compare Fig. S2E to F).

**Altered expression of limb patterning genes in β-catenin knockdown embryos**

The striking morphological changes of forelimb autopodia upon knockdown of β-catenin triggered us to analyze the expression of genes that are involved in the patterning of these structures. Two major signaling centers, the ZPA and the AER, control the morphogenesis of a limb. Hereby, Wnt3a stimulates the release of Fgf4 and Fgf8 from the AER. The Fgfs then induce the ZPA on the posterior end to produce Shh, which via Gremlin1 (Grem1) maintains Fgf expression in the AER. The Shh gradient further governs anterior-posterior patterning. Wnt7a and En1 secreted from the dorsal and ventral ectoderm, respectively, regulate dorsal-ventral patterning. We found in our analysis of E17.5 forelimb autopodia that the mRNA expression levels of all above mentioned genes were significantly reduced in β-catenin knockdown embryos except Wnt7a, whose expression was unchanged (Fig. 2). We could not detect Fgf8 expression in our samples, which might be due to the already advanced development of the autopodia used in our analysis. In summary, our findings show that anterior-posterior and dorsal-ventral patterning is altered in β-catenin knockdown embryos.

**Normal calcification of proximal forelimb bone primordia in β-catenin knockdown embryos**

Apart from the morphological changes of the forelimb autopodia, we could not detect differences in the morphology and mineralization of stylopodia and zeugopodia between control and β-catenin knockdown embryos in the whole mount skeletal preparation. Therefore, we decided to analyze ulna and radius in more detail histologically and histomorphometrically. To this end, forelimbs were collected at E18.5 and stained for calcification using the von Kossa method (Fig. 3A,B). Histomorphometrical quantification of the von Kossa stained area of zeugopodial bones revealed no significant difference between controls and knockdowns (Fig. 3C), showing that the ossification of ulna and radius was not disturbed in β-catenin knockdown embryos.

**Primary β-catenin knockdown rib chondrocytes behave normally upon stimulation of canonical Wnt signaling**

Since the calcification of bone primordia seemed to be intact in β-catenin knockdown embryos, we wondered whether the regulatory network underlying bone development was affected by the knockdown of β-catenin. To this end, we isolated primary rib chondrocytes and analyzed the expression of several genes with a role in cartilage and bone development. Genes of interest were transcription factors that are influenced by Wnt signaling in cartilage (Sox9) or bone (Runx2, Sp7 and Atf4) and extracellular matrix proteins specific for cartilage (type II collagen alpha 1–Col2a1 and Aggrecan–Acan) or bone (integrin-binding sialoprotein–Ibsp). Cultures of primary rib chondrocytes derived from control or β-catenin knockdown embryos showed no morphological differences (Fig. S3) despite the reduction of β-catenin (Ctnnb1) mRNA expression to 21% in knockdown cells (Fig. 4A). In unstimulated cells, the mRNA expression levels of the cartilage-specific genes Sox9, Col2a1 and Acan were significantly reduced in β-catenin knockdown cells, whereas the transcripts of the bone-specific genes Runx2, Sp7, Atf4 and Ibsp were similarly expressed in both cell lines (Fig. 4B). Additional Wnt3a stimulation led to a further reduction in the mRNA level of chondrocyte-specific genes regardless of the genotype (Fig. 4C,D). On the other hand, Runx2 mRNA expression levels did not change upon activation of canonical Wnt signaling, whereas Atf4 mRNA expression decreased by approximately 35% in control and β-catenin knockdown cells. Interestingly, Sp7 transcripts increased slightly in treated control cells (Fig. 4C), but did not change in knockdown cells upon Wnt3a stimulation.

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treatment (Fig. 4D). *Ibsp* was the only gene, whose mRNA expression level strongly increased in both cell lines upon Wnt3a stimulation. In aggregate, *β*-catenin knockdown primary chondrocytes already had a more bone-specific basic expression profile before Wnt3a stimulation compared to control primary chondrocytes. However, Wnt3a treatment almost completely abrogated chondrocyte-specific gene expression in both cell lines and induced the expression of the osteoblast-specific extracellular matrix component *Ibsp*. This shows that despite reduced *β*-catenin levels, knockdown cells are still able to respond normally to canonical Wnt signaling.

**DISCUSSION**

Reducing *β*-catenin expression to 25% has profound effects on the development of the limbs, spine and tail. Given the multiple roles of canonical Wnt signaling in the formation, patterning and maintenance of bones (Capdevila and Izpisúa Belmonte, 2001; Duan and Bonewald, 2016), our approach helps to identify the morphogenetic processes that can proceed normally even at low *β*-catenin levels, in contrast to those that require high *β*-catenin dosages.

**Defects due to impaired outgrowth and induction**

The most striking morphological findings in our mice were the absence of hindlimbs and the shortened, crooked tail, most likely resulting from impaired outgrowth of these structures. A human genetic syndrome that is characterized by the absence of all four limbs (Tetra-amelia syndrome) results from the autosomal recessive inactivation of the *WNT3* gene (Niemann et al., 2004). In mice, *Wnt3a* knockout leads to the absence of hindlimbs, deformed forelimbs and misshaped tails (Takada et al., 1994). Furthermore, in *Wnt5a* knockout mice the outgrowth of limbs and tail is severely compromised (Yamaguchi et al., 1999). However, whereas the loss of *Wnt3a* or *Wnt5a* produces a very specific upstream block of canonical Wnt signaling at the level of ligand-receptor interaction, the phenotype of our mice results from inadequate transduction of all canonical Wnt signals at the level of target gene transactivation. In more detail, *Wnt3* is centrally involved in the establishment and maintenance of the AER via the activation of the Wnt target gene *Fgf8* (Barrow et al., 2003; Kengaku et al., 1998; Narita et al., 2007). The AER is the master organizer of the growing limb, regulating the generation and allocation of progenitor cells to the future limb bones. Loss of AER function leads to impaired proliferation of progenitor cells and thus the premature termination of limb development (Lu et al., 2008). We previously showed that reduced *β*-catenin expression leads to diminished *Fgf8* expression levels in the tailbud (Rudloff and Kemler, 2012). Moreover, correct Fgf expression is required for the maintenance of a stem cell population of mesodermal cells that contributes to the growth of the embryo (Boulet and Capecchi, 2012). Of note, the teratogenic effect of thalidomide, which leads to deformed limbs, is caused by the untimely inhibition of canonical Wnt signaling and increased cell
death (Knobloch et al., 2007). Therefore, the proper induction and outgrowth of tail and limbs not only depends on the integrity of the Wnt-Fgf axis, but also on the expression level of β-catenin. As shown in this study, 25% β-catenin expression levels are not sufficient for the maintenance of this signaling axis, probably due to the early exhaustion of the proliferation potential of progenitor cells. This also offers an explanation as to why hindlimbs show a higher degree of distortion than forelimbs. Since the cells in the caudal embryo have passed through more cells divisions before they become part of the future hindlimb, they might not anymore be able to generate enough cells to form a limb.

**Defects due to impaired patterning**

Although forelimbs are present in β-catenin knockdown embryos, their structure is abnormal; the autopodias, and rarely the zeugopodium, also show irregularities. In the forelimbs the phenotype is highly variable, ranging from the addition of multiple irregularly shaped digits within the plane of the paw and protruding the paw at anomalous angles, to reduced numbers of phalanges or the absence of one of the two zeugopodial bones. Specification of the anterior-posterior identity of the bones of zeugopodium and autopodium is achieved by a Sonic hedgehog (Shh) morphogen gradient, originating from the zone of polarizing activity (ZPA), located at the posterior end of the growing limb (Capdevila and Izpisúa Belmonte, 2001; Chiang et al., 2001). Canonical Wnt signaling has also been shown to be important for the maintenance of the ZPA in concert with the AER (Pearse and Tabin, 1998). Hereby, Fgf5s from the AER form a positive feedback loop involving Shh and the Bmp inhibitor Gremlin1, which in turn sustain the β-catenin activation required for the maintenance of the AER. The coordinated activity of ZPA and AER is further underlined by common enhancers that regulate the expression of important patterning genes from both signaling centers (VanderMeer et al., 2014). In our β-catenin knockdown embryos all members involved in the feed forward loop between AER and ZPA are diminished. Thus, our findings do not allow us to draw definite conclusions on whether the patterning activity of the ZPA functions normally with reduced β-catenin levels, since the AER defects might obscure ZPA-dependent phenotypes. On the other hand, the observed patterning defects might arise from small local differences in Shh expression. A more specific knockdown of β-catenin in the AER or ZPA is needed to clarify this question.

**Differentiation of cartilage and bone**

There are several steps during skeletogenesis where Wnt/β-catenin signaling has an inhibiting effect, while at others activating effects predominate. Early in osteogenesis, cells forming the skeletal primordia differentiate into chondrocytes or osteoblast precursors. In this process, canonical Wnt signaling directly promotes the generation of osteoblast precursor cells, marked by Runx2 and Col2a1 (Regard et al., 2012). In contrast, blocking Wnt signaling at this step favors the differentiation into Sox9-positive chondrocytes (Bennett et al., 2005; Huang et al., 2007). Osteoblast precursors themselves are prevented from differentiation into chondrocytes by canonical Wnt signaling. Conditional bone-specific loss of β-catenin during early development has been shown to lead to abnormalities in osteoblastogenesis and ectopic development of cartilage (Day et al., 2005; Hill et al., 2005). The primary chondrocytes isolated from β-catenin knockdown embryos in our study showed a β-catenin expression level of approximately 25% compared to wild-type cells. This corresponds exactly to the expected expression level (Rudloff and Kemler, 2012) and confirms the usability of our mouse model. Based on the literature, we expected that the knockdown of β-catenin would also lead to increased cartilage differentiation and a decreased number of osteoblasts, and subsequently a reduction in calcified bone. However, to our surprise, the histomorphometrical analysis of forelimb zeugopodia showed no significant difference in the mineralized bone area between wild-type and mutant embryos. Moreover, the expression of cartilage-specific genes was lower in primary β-catenin knockdown cells than in control cells. These findings suggest that β-catenin knockdown does not favor the formation of additional cartilage. In agreement with this, canonical Wnt signaling was also described to have an inhibitory effect on the transition from early osteoblasts to mature osteoblasts. Therefore, an early potential developmental bias towards cartilage differentiation in our knockdown embryos could be counterbalanced by an accelerated maturation of osteoblasts at later stages. Furthermore, stimulation of canonical Wnt signaling almost completely abolished the expression of cartilage markers and significantly increased the expression of the bone-specific matrix protein Ibsp in both cell types. Based on these findings our isolated cells most likely correspond to a mix of chondrocytes and osteoblast precursors, which, after Wnt3a treatment, mature into Ibsp-positive early osteoblasts (Regard et al., 2012). Moreover, 25% of β-catenin expression seems to be sufficient to sustain normal mineralization of bone.

**Conclusions and outlook**

Our study demonstrates that β-catenin expression levels as low as 25% are sufficient for the correct differentiation of cartilage and bone in skeletal primordia, but not sufficient for the correct patterning of limbs and tail (summarized in Table 1). Thus, the phenotype of our mutant mice most likely results from incorrectly specified signaling centers such as AER and ZPA together with a reduced proliferative capacity of skeletal progenitor cells, but not from differentiation defects of chondrocytes and osteoblasts. Our findings also shed more light on β-catenin dependent processes during bone regeneration and fracture repair. During bone repair, similar mechanisms are at work as during embryonic development. Here, mutations increasing β-catenin levels show an accelerated repair process (Arioka et al., 2013), but also lead to unwanted side effects such as higher bone mass or the formation of excess fibrous tissue as in pseudarthrosis or osteoarthritis (Ghadakzadeh et al., 2016; Tornero-Esteban et al., 2015). Conversely, loss of canonical Wnt signaling impairs the fracture healing process (Burgers et al., 2016; Huang et al., 2012). Since the level of Wnt proteins declines with age (Rauner et al., 2008), treatment of low bone mass in elderly patients with novel Wnt agonists such as antibodies against Sclerostin or Dickkopf will become more and more important.

| Table 1. Summary of skeletal defects in β-catenin knockdown embryos |
|-----------------------------|------------------|------------------|------------------|
| Induction                  | Front limbs       | Hindlimbs        | Tail             |
| Outgrowth                  | Yes              | Yes              | Yes              |
| AER                        | Mostly intact     | Truncated        | Ring-tail        |
| ZPA                        | Disturbed         | Disturbed        | -                |
| Tailbud                    | -                | -                | -                |
| Patterning                 | Disturbed distally| Disturbed proximally| Disturbed        |
| Stylopodium                | Normal            | Mostly absent    | -                |
| Zeugopodium                | Mostly normal     | Absent           | -                |
| Autopodium                 | Disturbed         | Absent           | -                |
| Mineralization             | Yes              | Partially        | Not observed     |
The experimental procedures were approved by the committee for animal experimentation of the canton Bern. All procedures involving experimental animals were performed in compliance with local animal welfare laws, guidelines and policies. The following genetic traits were combined on a C57BL/6×129S6/SvEvTac background: (1) Cre-recombinase under the control of the caudal type homeobox promoter (Cdx1::Cre) (Hierholzer and Kemler, 2009); (2) a conditional β-catenin allele, in which exons 2-6 are flanked by loxp sites (β-catenin flox/flox) (Braut et al., 2001); (3) β-catenin under control of the ROSA26 promoter (ROSA26::β-catenin) (Rudloff and Kemler, 2012). In (Cdx1::Cre)β-catenin flox/flox;ROSA26::β-catenin mice, the endogenous β-catenin will be deleted in the caudal half of the embryo and replaced by the ROSA26 transgene. Mice that express β-catenin from both ROSA26 loci retain approximately 25% of β-catenin compared to wild types. In this study, embryos of both sexes were analyzed at E17.5 and E18.5. Genotyping of endogenous β-catenin was performed as previously described (Braut et al., 2001).

Histology, skeletal staining and micro CT imaging

For Hematoxylin-Eosin (HE) staining, paraffin embedded tissues were cut into 7 μm sections and rehydrated. Sections were then incubated in 1:10 dilution of Harris hematoxylin solution modified (Sigma-Aldrich, Buchs, Switzerland) with the following parameters: X-ray source (55 kVp with 145 mA at medium resolution), diameter of sample holder (16 mm), acquisition: U.H.-D. and acceptance level: U.H.-D. The results of qRT-PCR and von-Kossa stained area were tested for significance using unpaired, two-tailed Student’s t-tests. P-values <0.05 were considered statistically significant.

Acknowledgements

We thank Mark Siegrist for his excellent technical assistance with the micro CT scans and Dr Thomas Hammond for critically reading the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.R.; Methodology: S.R.; Software: T.P.; Validation: T.P., S.R.; Formal analysis: T.P., S.R.; Investigation: T.P., S.R.; Data curation: T.P., S.R.; Writing - original draft: T.P.; Writing - review & editing: S.R.; Visualization: S.R.; Supervision: U.H.-D., S.R.; Project administration: U.H.-D., S.R.; Funding acquisition: U.H.-D.

Funding

This work was supported by the NCCR Kidney.CH program (Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung).

Supplementary information available online at http://bio.biologists.org/lookup doi:10.1242/bio.023572.supplemental

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Received 1 May 2020 Revised 31 July 2020 Accepted 14 August 2020

Downloaded from http://bio.biologists.org/ at University of Bern on October 1, 2019
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