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Osteoblast-targeted disruption of glucocorticoid signalling does not delay intramembranous bone healing

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ABSTRACT

Objective: Glucocorticoids at pharmacological doses have been shown to interfere with fracture repair. The role of endogenous glucocorticoids in fracture healing is not well understood. We examined whether endogenous glucocorticoids affect bone healing in an *in vivo* model of cortical defect repair.
Methods: Experiments were performed using a well characterised mouse model in which intracellular glucocorticoid signalling was disrupted in osteoblasts through transgenic overexpression of 11β-hydroxysteroid-dehydrogenase type 2 (11β-HSD2) under the control of a collagen type I promoter (Col2.3-11β-HSD2). Unicortical bone defects (⌀0.8 mm) were created in the tibiae of 7-week-old male transgenic mice and their wild-type littermates. Repair was assessed via histomorphometry, immunohistochemistry and microcomputed tomography (micro-CT) analysis at 1–3 weeks after defect creation.
Results: At week 1, micro-CT images of the defect demonstrated formation of mineralized intramembranous bone which increased in volume and density by week 2. At week 3, healing of the defect was nearly complete in all animals. Analysis by histomorphometry and micro-CT revealed that repair of the bony defect was similar in Col2.3-11β-HSD2 transgenic animals and their wild-type littermates at all time-points.
Conclusion: Disrupting endogenous glucocorticoid signalling in mature osteoblasts did not affect intramembranous fracture healing in a tibia defect repair model. It remains to be shown whether glucocorticoid signalling has a role in endochondral fracture healing.

1. Introduction

Glucocorticoids (GC) are widely used in almost all fields of medicine. They have been proven to be of great benefit to patients suffering from systemic inflammatory diseases, malignancies or transplant rejection. It is, however, well established that GC at pharmacological doses exert detrimental effects on bone, muscle, cartilage and skin. Thus, up to 50% of patients receiving chronic GC therapy will suffer from fragility fractures [1]. Moreover, fracture healing may be significantly impaired in patients on high-dose ("exogenous") GC treatment [2]. In contrast, *in vitro* [3–7] and *in vivo* studies [7] suggest that GC at *physiological* ("endogenous") levels have anabolic effects on bone cells by promoting osteoblast differentiation and matrix mineralisation. However, the role of endogenous GC on bone healing is unknown.

In the current study, we made use of a transgenic mouse model in which the expression of a GC-inactivating enzyme, 11β-hydroxysteroid-dehydrogenase type 2 (11β-HSD2), has been targeted exclusively to osteoblasts and osteocytes using the 2.3 kb col1agen type Ia1 promoter [8]. Under physiological conditions, 11β-HSD2 is mostly expressed in the kidney but absent from skeletal tissues. Accordingly, 11β-HSD2 is not found to be expressed in the calvaria, femur or vertebra of non-transgenic (WT) littermates [6,7,9,11].

Targeted overexpression of 11β-HSD2 in Col2.3-11β-HSD2 tg mice represents a highly specific means of disrupting GC signalling in mature osteoblasts and osteocytes [7,9]. This model therefore provides a unique opportunity to investigate the role of endogenous GC signalling in bone development, maintenance and repair. In our previous studies using this transgenic mouse model, we have shown that endogenous GC signalling in mature

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osteoblasts is necessary for proper intramembranous bone development in the prenatal and neonatal calvaria [7]. At the adult stage, we found that transgenic mice were characterised by lower bone volume, lower trabecular number and higher trabecular separation in tibial trabecular bone, as well as lower tibial cortical bone area and periosteal and endosteal perimeters. These changes resulted in a marked decrease in mechanical bone strength and stiffness [11]. Taken together, we have demonstrated that endogenous glucocorticoids are required for normal bone development and maintenance.

The current study was designed to investigate the role of GC in osteoblastic repair. To this aim, a well defined intramembranous fracture model (in which bone formation is induced without an endochondral component) was applied to Col2.3-11β-HSD2 tg mice and their WT littermates, and time-dependent structural and histological parameters of bone repair were studied as main outcomes.

2. Materials and methods

2.1. Experimental animals

Col2.3-11β-HSD2 tg mice were generated as described previously [9] and were provided as a gift by Dr. Barbara Kream (Department of Medicine, University of Connecticut Health Center, Farmington, CT, USA). Mice were maintained at the animal facilities of the ANZAC Research Institute (Sydney, Australia) in accordance with Institutional Animal Welfare Guidelines and according to an approved protocol.

2.2. Tibia cortical defect repair model

Surgical procedures were performed on 7-week-old male Col2.3-11β-HSD2 tg mice and their WT littermates. Under anaesthesia and analgesia, an incision was made over the dorsal aspect of the knee and the tibia was exposed by dividing the anterior tibial muscle. A unicortical tibial bone defect was created 5 mm below the tibia patellar tendon insertion using a 0.8 mm diameter drill. Subsequent to the operation, X-rays were obtained by Faxitron to confirm the correct position of the defect. Animals were allowed unrestricted cage activity and were closely monitored for pain-related behaviour. Fracture healing was assessed at 1–3 weeks following generating of the cortical defect.

2.3. Sample preparation

Mice were sacrificed at the assigned time-points by cervical dislocation under anaesthesia. Tibiae were collected and fixed in 4% paraformaldehyde/PBS for 48 h at 4 °C. After micro-CT imaging, samples were decalcified in 10% EDTA, pH 7.0, for 3 weeks at 4 °C with twice weekly change of the solution. Tibiae were then processed for paraffin embedding. Serial 5 μm sections were obtained and stained with hematoxylin and eosin (H&E). To identify osteoclasts, sections were stained for tartrate resistant acid phosphatase (TRAP) using naphthol AS-BI phosphate (Sigma) as a substrate and fast red violet Luria–Bertani salt (Sigma) as a detection agent for the reaction product [10].

2.4. Microcomputed tomography (micro-CT)

Micro-CT analyses of tibiae were conducted with a SkyScan 1172 scanner (SkyScan, Kontich, Belgium). Scanning was done at 100 kV, 100 μA using a 1 mm aluminium filter with an exposure set to 590 ms. In total, 1800 projections were collected at a resolution of 6.93 μm/pixel. Sections were reconstructed using a modified Feldkamp cone-beam algorithm with beam hardening correction set to 50% [11]. VGStudio MAX 1.2 software (Volume Graphics GmbH, Heidelberg, Germany) was used to obtain 3D visualisation from reconstructed sections. CTAnalyser software (ver. 1.02) (SkyScan) was employed to analyse the defect callus in a region of interest defined manually. A region of interest was selected which covered the entire volume of the immediate cortical defect site. Separation of bone tissue from other soft tissue was ensured by applying segmentation thresholds. The percentage of callus bone volume (BV/TV) and its mean density was analysed.

2.5. Histomorphometry

Histomorphometric characterisation of defect calluses was performed on all defect tibiae using Bioquant Osteo II System (Bioquant, Nashville). Three representative levels of H&E and TRAP stained sections, each approximately 15 μm apart, were analysed. The selected region of interest in the defect area was a rectangle, with a standard height of 350 μm and a width adjusted to the defect dimension with a gap of 30 μm to each side of the original cortical bone. Bone surfaces covered with osteoblasts (Ob.S/BS) and osteoclasts (Oc.S/BS) were measured manually for the region of interest at 200× magnification. Likewise, number of osteoclasts (N.Oc/BS) was counted and the volume of the newly formed bone (BV/TV) was analysed [11].

2.6. Immunohistochemistry

Immunohistochemical assessment of 11β-HSD2 transgene expression at the defect site was performed on 5 μm thick tibiae sections of Col2.3-11β-HSD2 tg and WT samples. The polyclonal rabbit anti-rat 11β-HSD2 antibody RAH23 was used as the primary antibody [7,12] and a biotinylated goat anti-rabbit immunoglobulin (Vectastatin ABC kit, Vector Laboratories, Burlingame, USA) served as the secondary antibody. DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, USA) was used for chromagen development. Samples were counterstained with Gill's hematoxylin [7].

2.7. Statistical analysis

Data are represented as the means ± standard error of the mean (SEM). Statistical analysis was performed with Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

Surgical procedures were well tolerated by all animals. Mice did not exhibit any abnormal behaviour and showed no preference in the use of their legs throughout the post-operative monitoring period.

Cortical defects were created in a reproducible manner and over time were restored to their original architecture via intramembranous bone formation. At week 1, micro-CT imaging demonstrated formation of poorly mineralized bone at the drill site and bone marrow cavity, while histology revealed active woven bone formation with bone surfaces densely lined with osteoblasts (Fig. 1A). Deposition of new bone continued throughout week 2, forming a bony bridge at the drill site (Fig. 1B). At week 3, bone healing was nearly complete. Thus, the defect was no longer apparent on micro-CT imaging as it was filled with tissue indistinguishable from the surrounding cortical bone (Fig. 1C). However, original lamellar and newly formed woven bone was clearly discernible by polarized light microscopy of histological sections, again demonstrating that the defect was nearly completely filled with new bone (Fig. 1C). Throughout the repair process, expression of 11β-HSD2 was monitored by immunohistochemistry. As expected, 11β-HSD2

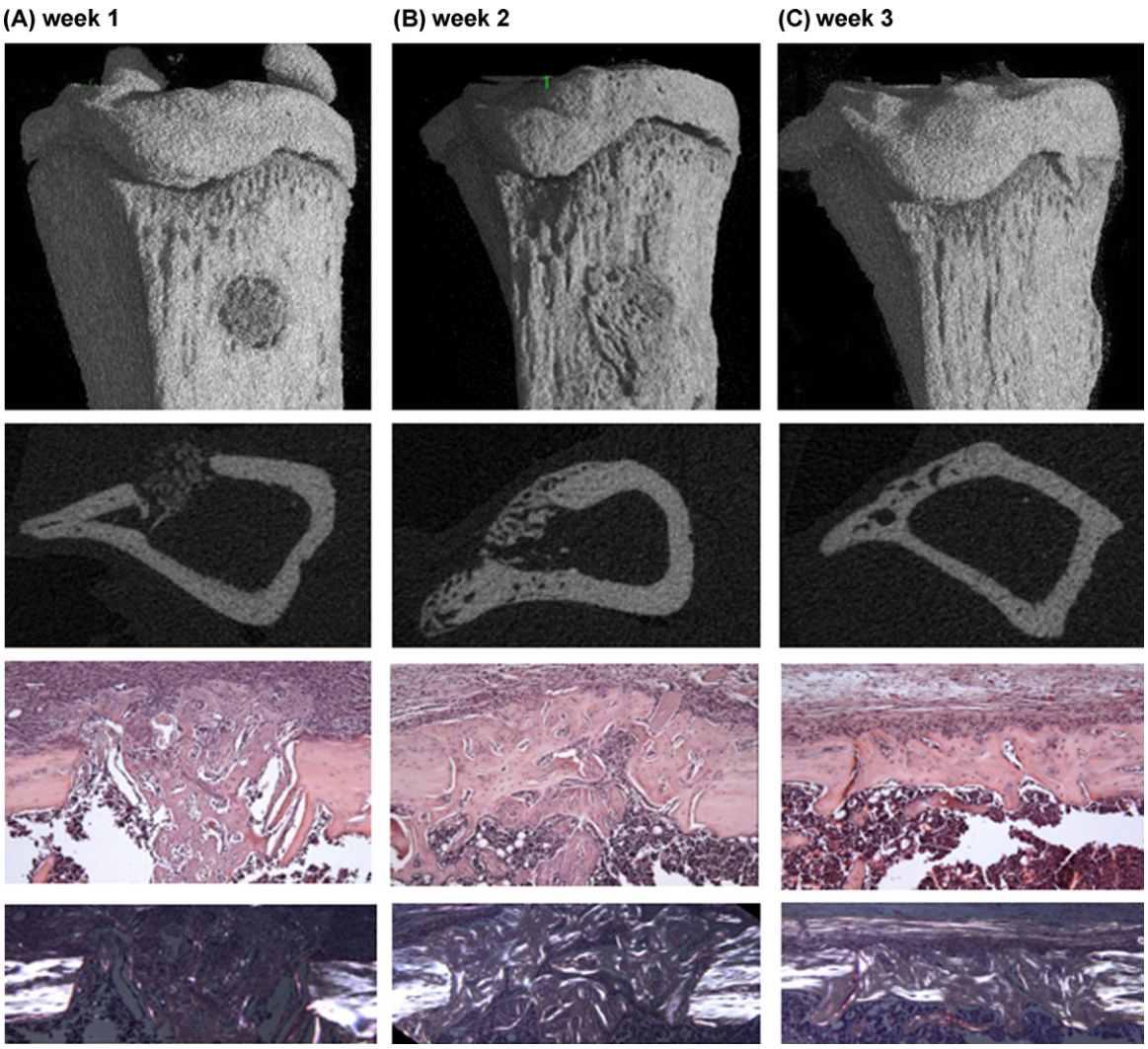

Fig. 1. Micro-CT images (3D, first panel; cross-sections, second panel) and histology (H&E, 100× magnification, third panel; same sections under polarized light microscopy, fourth panel) of unicortical diaphysal defects in the proximal tibia of WT animals at 1–3 weeks post-fracture. Morphological comparison of the defect site revealed no difference between WT and tg animals at respective time-points. At week 1 (A), mineralized woven bone was formed at the defect site, which increased in volume and density by week 2 (B). At week 3 (C), healing of the defect was nearly completed in all WT and tg animals.

was detected in osteoblasts and osteocytes of Col2.3-11β-HSD2 tg animals (Fig. 2A) but not in WT mice (Fig. 2B).

3.1. Microcomputed tomography

Micro-CT analysis was performed on all samples at week 1 and 2 post-fracture to characterize the newly formed bone (week 1: WT $n=12$, tg $n=14$; week 2: WT $n=13$, tg $n=13$). Quantification of percentage of newly formed bone volume (BV/TV) in the defect area and its mean density revealed no statistical difference between WT and tg animals at both time-points ($p>0.05$; Fig. 3A and B). Samples obtained at week 3 (WT $n=9$, tg $n=9$) were not analysed as the fracture site could not be accurately identified anymore by this technique.

3.2. Histomorphometry

Histomorphometric measurements were performed to characterize bony repair on the cellular level. Tibia samples (week 1: WT $n=12$, tg $n=14$; week 2: WT $n=13$, tg $n=13$; week 3: WT $n=9$,

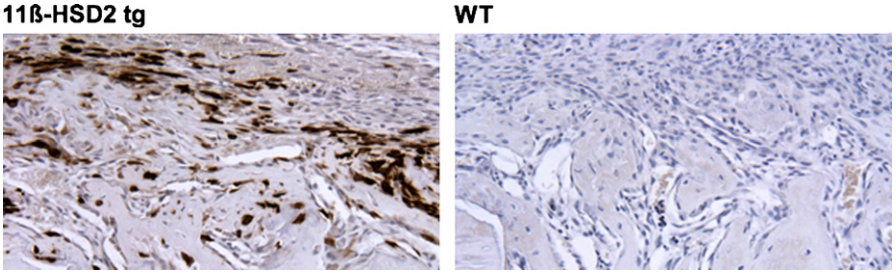

Fig. 2. Immunohistochemical assessment of the defect site showed 11β-HSD2 expression in transgenic samples (A). No transgenic activity was detected in WT samples (B) (200× magnification).

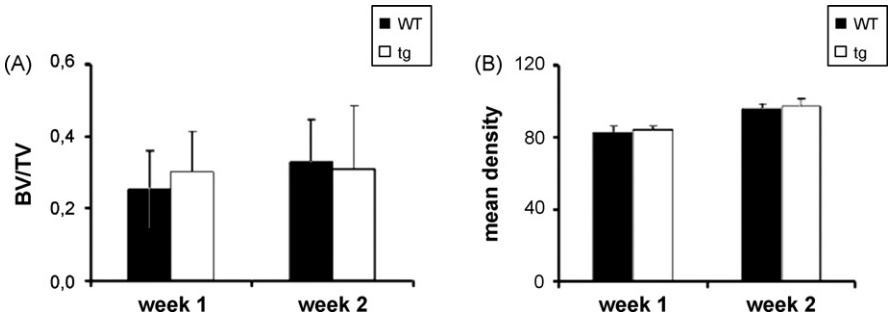

Fig. 3. Micro-CT analysis of immediate defect site, excluding cortical bone. Comparison of WT and tg animals at week 1 and 2 post-surgery, revealed similar results for (A) % of newly formed bone volume (BV/TV) and (B) mean density of bone volume ($p > 0.05$). At week 3, an accurate region of interest was not detectable, due to progressed tissue repair. Shown are means ± SEM.

tg $n = 9$) taken at all time-points were analysed. The percentage of bone surface covered with osteoblasts (Ob.S/BS) and osteoclast (Oc.S/BS), and osteoclast number per bone surface (N.Oc/BS) did not differ between tg and WT mice at any time-point ($p > 0.05$, Fig. 4C–E). Furthermore, percentage of bone volume (BV/TV) and percentage of bone surface per bone volume (BS/BV) were similar between the two groups at all time-points (Fig. 4A and B). Two-way ANOVA showed no interaction between time-point and genotype for any of the analysed parameters.

4. Discussion

Intracellular GC metabolism is locally modulated at the pre-receptor level by two isoforms of the 11β-hydroxysteroid-dehydrogenase (11β-HSD) family of enzymes: while 11β-HSD type 1 predominantly converts inactive GC to their biological active forms [13,14], 11β-HSD type 2 uni-directionally catalyses the conversion of active GC to their inactive metabolites [13]. Under physiological conditions, 11β-HSD type 2 is expressed exclusively in mineralocorticoid-rich tissues such as the kidney, where it protects the mineralocorticoid receptor from illicit GC binding. In the tg mouse model used in this study the rat gene for 11β-HSD type 2 was linked to the 2.3 kb collagen type I (Col2.3) promoter to target overexpression of 11β-HSD2 specifically to mature osteoblasts and osteocytes [6,7,9,15]. The resulting transgenic animal ('Col2.3-11β-HSD2 tg') shows complete disruption of intracellular GC signalling in mature osteoblasts and osteocytes [6,7,9]. As 11β-HSD2 is normally not detectable in osteoblasts [6,7], transgenic expression of this enzyme in osteoblasts provides a highly specific tool to investigate the physiological role of endogenous GC signalling in osteoblast differentiation and function.

Previous *in vivo* experiments employing the same transgenic mouse model suggested not only a role of endogenous GC in trabecular bone growth and maintenance of bone mass in adult mice but also in cortical bone mass acquisition [9,11]. In particular, the long bones derived from adult Col2.3-11β-HSD2 tg mice had significantly reduced bone strength and bone volume when compared to long bones of their wild-type (WT) littermates [11].

We chose the intramembranous fracture healing model since bone formation and remodelling occurs without an endochondral intermediate and thus, both the level of communication between mesenchymal stem cells and mature osteoblasts, and the magnitude of effects on osteoblast function are likely to be greater due to accelerated bone metabolism occurring in fracture repair.

The tibial cortical defect repair model requires a technically relatively simple operating procedure, which was well tolerated by all animals as indicated by the lack of pain-related behaviour throughout the post-operative monitoring period. Moreover, defects were created in a reproducible manner. Several studies have employed cortical defect drill hole models in mice [16–19] and similarly

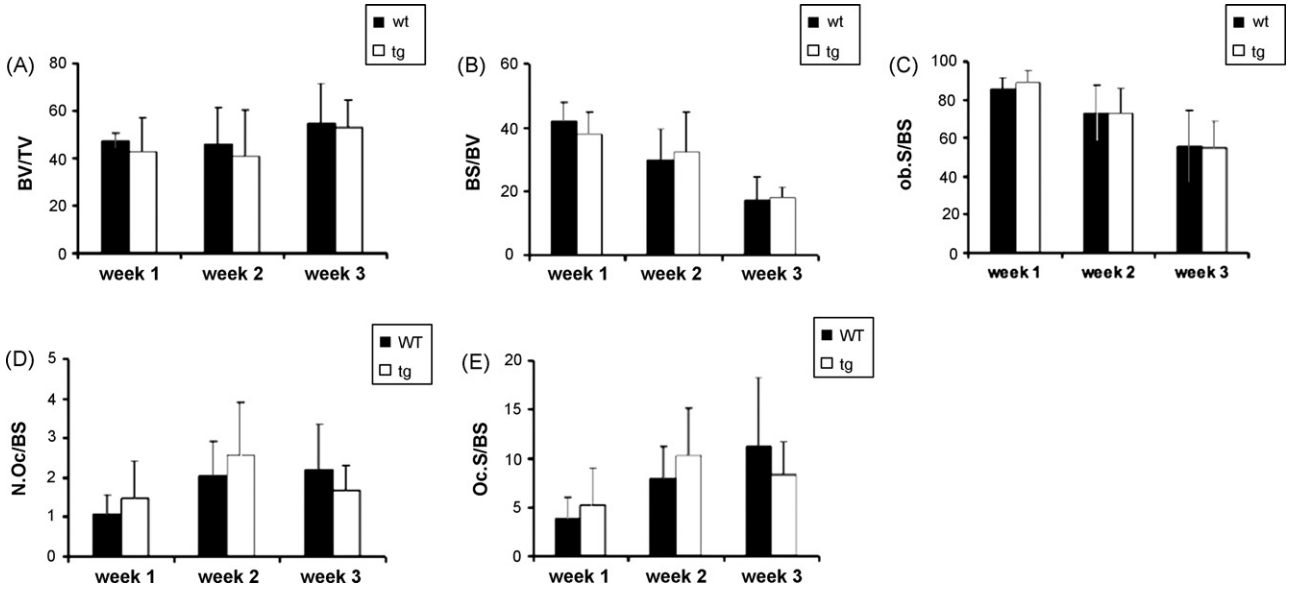

Fig. 4. Histomorphometry analysis of H&E and TRAP stained tibia samples at 1–3 weeks post-fracture. No difference between WT and tg animals was observed in (A) % bone volume (BV/TV), (B) % bone surface (BS/BV), (C) % osteoblast surface (Ob.S/BS), (D) number of osteoclasts/mm^2 (N.Oc/BS) and (E) % of osteoclast surface (Oc.S/BS) ($p > 0.05$). Shown are means ± SEM.

observed advantages such as reduced pain, lack of infections [16], reproducibility of defects and excellent toleration of the procedure by the animals [18]. However, these studies differ in details of experimental design, including the strain of mice used, the size of the defect produced and the duration of the monitoring period.

In the current study, analysis of the repair callus by histomorphometry and micro-CT revealed no difference between tg animals and their WT littermates in regards to both cellular and structural characteristics of bone healing. These results suggest that endogenous GC signalling does not affect intramembranous fracture repair in this model and mouse strain.

The Col2.3-11β-HSD2 tg mouse model has been well characterized in previous studies and the activity of the 11β-HSD2 transgene was confirmed by measuring the conversion of (^{3}H) corticosterone to 11-dehydrocorticosterone *in vitro* [9]. Immunohistochemical analyses performed in this and previous studies demonstrate that transgenic protein expression is specifically localized to osteoblasts and osteocytes of cortical and trabecular bone in transgenic mice [9]. In the tibial cortical defect repair model described here, immunohistochemistry confirmed the expression of 11β-HSD2 transgene in osteoblasts and osteocytes in the repairing bone defect site. Transgene activity was not detected in WT animals.

Our observations raise a number of possibilities in regards to the underlying physiological mechanisms. Endogenous GC signalling in mature osteoblasts may not possess a non-redundant role in intramembranous bone repair, and their functions in regards to new bone formation or bone repair may be shared by other signalling molecules or pathways. During the intramembranous bone repair osteoblasts differentiate directly from mesenchymal cells, whereas endochondral bone repair involves a cartilage intermediate [20,21]. However, since this intramembranous bone was created in an endochondral bone environment, the process of intramembranous bone formation may differ from that occurring in the calvaria.

As with all transgenic models, there is the possibility that adaptation to the impaired GC signalling in mature osteoblasts and osteocytes occurred in the adult tissue. However, as intramembranous fracture healing is initiated by mesenchymal stem cells recruited and completing differentiated into osteoblasts over a very short (2 weeks) time frame, our observations are unlikely to be related to adaptation.

In conclusion, our results suggest that endogenous GC signalling in mature osteoblasts is not essential for fracture healing in this model of defect repair. However, it remains to be shown whether GC plays a role in endochondral fracture repair and in intramembranous defect repair occurring in an intramembranous environment.

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