Human Sex Matters: Y-linked lysine demethylase 5D drives accelerated male osteogenic differentiation

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Running Head: KDM5D drives sexually dimorphic osteogenic differentiation

Abstract

Female sex is increasingly associated to a loss of bone mass during aging and an increased risk for fractures developing nonunion. Hormonal factors and cell-intrinsic mechanisms are suggested to drive these sexual dimorphisms, although underlying molecular mechanisms are still a matter of debate. Here, we observed a decreased capacity of calvarial bone recovery in female rats and a profound sexually dimorphic osteogenic differentiation human adult neural crest-derived stem cells (NCSCs). Next to an elevated expression of pro-osteogenic regulators, global transcriptomics revealed Lysine Demethylase 5D (KDM5D) to be highly upregulated in differentiating male NCSCs. Loss of function by siRNA or pharmacological inhibition of KDM5D significantly reduced the osteogenic differentiation capacity of male NCSCs. In summary, we demonstrate craniofacial osteogenic differentiation to be sexually dimorphic with the expression of KDM5D as a prerequisite for accelerated male osteogenic differentiation, emphasizing the analysis of sex-specific differences as a crucial parameter for treating bone defects.

Introduction

The capacity for bone regeneration as well as the severity and occurrence of bone disorders is increasingly noticed to be linked to the sex of an individual (1), reviewed in (2). Here, particularly female sex is linked to a gradual loss of bone mass after the age of around 35 (3, 4) and considered as a major risk factor for decreased osseointegration of implants (5) and fractures developing nonunion (1, 6). Postmenopausal women also show an increased prevalence of osteoporosis compared to their male counterparts (7-9). While aggressive osteoclastic bone resorption was recently reported to be linked to age and menopausal status of female individuals (4), the bone formation rate simultaneously decreases (4, 9). On mechanistic level, estrogen was reported to regulate bone resorption and formation (10) and the osteogenic activity of stem cells in mouse osteoporosis models (11). However, Meszaros and colleagues reported a significantly increased regeneration of cranial bone defects in castrated or non-operated male mice compared to non-operated or ovariectomized female animals, suggesting the additional presence of non-hormonal mechanisms guiding sexual dimorphic bone regeneration rates (12). In this line, increasing evidences suggest that stem cell-intrinsic mechanisms account for sex-specific differences in bone recovery and occurrence of bone disorders (reviewed in (2)). For instance, male mouse skeletal muscle-derived stem cells were reported to reveal a greater amount of mineralization and ALP-activity after osteogenic differentiation compared to their female counterparts (13). In humans, male adipose tissue stem cells (ASCs) were likewise shown to more efficiently give rise to osteogenic derivatives compared to female ASCs (14). Despite these promising findings, the molecular mechanisms of the observed sex-specific differences in osteogenic differentiation of stem cells, particularly in the cranial and craniofacial region, are still a matter of debate.

Facing this challenge, the present study demonstrates sex-specific differences in the regeneration of critical size calvarial rat bone defects as well as in the osteogenic differentiation of adult human craniofacial stem cells and determines underlying molecular drivers regulating sex-specific stem cell behavior. We particularly utilized adult human neural crest-derived stem cells (NCSCs) from the nasal cavity as a human *in vitro* model for investigating sexual dimorphisms in craniofacial osteogenic differentiation in accordance to our previous studies (15-17). For guiding osteogenic differentiation of NCSCs, collagen type I fibers harboring 30 nm pores were applied as a pro-osteogenic biophysical cue (16). Next to collagen type I fibers, the clinically approved hemostatic collagen sponge Spongostan was utilized for investigating the sexual dimorphic regeneration of critical size calvarial rat bone defects. We recently demonstrated the distinct nano- and microtopography of Spongostan to be solely sufficient for guiding bone recovery in critical size calvarial bone defects in male rats (18, 19). Taking advantage of these biophysical cues driving osteogenic differentiation, we show here an elevated

regeneration of critical size calvarial bone defects in male rats after transplantation of collagen fibers or Spongostan compared to their female counterparts. In accordance to our observations *in vivo*, human NCSCs from female individuals revealed a strong delay in osteogenic differentiation on collagen type I fibers over time in comparison to NCSCs from male donors. Global gene expression profiling following osteogenic differentiation revealed a sex-specific expression pattern of negative osteogenic regulators like micro RNA 137 (MIR137HG) and taxilin gamma (TXLNG) in female NCSCs, while pro-osteogenic regulators including C-FOS and Cytochrome P450 Family 7 Subfamily B Member 1 (CYP7B1) were upregulated in male stem cells. We further functionally validated the expression of the Y-linked Lysine Demethylase 5D (KDM5D) as a relevant driver of the observed elevated osteogenic differentiation capacity of male craniofacial NCSCs.

Results

Calvarial critical size defects in female rats show impaired regeneration compared to male animals

We determined potential differences in bone recovery between the sexes by comparing regeneration of critical-size calvarial bone defects in male and female rats. To allow recovery of the lesion, we applied the collagen type I fibers or the collagen sponge Spongostan in accordance to our previous observations (18, 19) after trepanation of calvarial bones. Although no closures of the critical-size defects were observable in controls via micro-computed tomography (μ CT), we observed a significantly reduced bone mineral density (BMD) in female rats compared to male ones (Fig. 1A). A significantly decreased bone volume (BV) and BMD was further observable in female animals compared to their male counterparts after transplantation of collagen type I fibers (Fig. 1B) or Spongostan (Fig. 1C). In particular, a complete closure of the lesion was observable in Spongostan-treated male rats, while female rats revealed only partial recovery (Fig. 1C). Histological examination of the newly formed bone confirmed the observations made via μ CT, particularly revealing increased amounts of new formed bone in male rats compared to female animals after transplantation of Spongostan or collagen type I fibers (Fig. 1 D, stars indicate new bone). Thus, we demonstrate here a strong and sex-specific impairment of female rats in regenerating calvarial bone lesions compared to males.

Female adult human craniofacial stem cells show strongly delayed osteogenic differentiation

Next to assessing sex-specific differences in bone regeneration of calvarial lesions *in vivo*, we investigated potential sex-dependent differences in osteogenic differentiation in a human model system. We used human craniofacial NCSCs from both sexes and cultivated them on collagen fibers (Fig. 3A) to induce osteogenic differentiation by physical cues only (16). NCSCs from male individuals revealed a strong Alizarin Red S-stained calcium deposition indicating successful osteogenic differentiation after 30 days of culture (Fig. 3B). On the contrary, we observed no signs of Alizarin Red S-positive calcification in female NCSCs cultivated under the same conditions for 30 days (Fig. 3C). After 40 additional days (day 70) of culture on collagen type I fibers, NCSCs from female donors were also able to differentiate and showed Alizarin Red S-stained calcium deposits (Fig. S1). In summary, adult NCSCs from female individuals differentiated on collagen type I fibers but revealed a strong delay in osteogenic differentiation over time in comparison to male NCSCs (under physiological differentiation cues without hormones).

expression of common osteogenic markers like ALP, Runx2 and Osteonectin in male and female NCSCs after 14 and 30 days, while transcripts solely expressed in either male or female NCSCs belonged to similar GO-terms.

Transcriptome analysis reveals an extreme sexually dimorphic gene expression

To determine the molecular basis of their sexually dimorphic differentiation behavior, RNA-sequencing of adult human craniofacial NCSCs was performed after 14 and 30 days of osteogenic differentiation. Global gene expression profiling revealed the expression of already known osteogenic markers like ALP, Runx2 and Osteonectin in male and female NCSCs after 14 and 30 days of differentiation without observable differences between the sexes (Fig. 2D-E). We found 507 (14 days) and 887 (30 days) transcripts solely expressed in male NCSCs, while 543 (14 days) and 454 (30 days) were exclusively present in female NCSCs upon differentiation (Fig. 2D-E). Interestingly, we observed strong differences in the percentages of genes belonging to the molecular function GO-terms “binding” and “molecular function regulator”, which were elevated in male NCSCs compared to female ones after 14 days of differentiation (Fig. 2D). On the contrary, the percentages of genes belonging to the molecular function GO-term “transporter activity” was found to be increased in female NCSCs (Fig. 2D). We further observed nearly similar amounts of molecular function-related genes after 30 days of differentiation between male and female NCSCs (Fig. 2E). Likewise, female and male NCSCs revealed nearly similar percentages of expressed genes belonging to biological process GO terms like “biological adhesion”, “biological regulation”, “cellular process”, or “response to stimulus” (Fig. 2D-E). These findings show that female NCSCs at least to some extent undergo osteogenic differentiation upon culture on collagen fibers despite the lack of calcium deposition after 30 days.

On the contrary, in male NCSCs differential gene expression analysis revealed a significantly elevated expression of characteristic osteogenic markers and regulators like BMP4, MSX2, C-FOS, COL4A4, COL11A1 and ITGA1 (table 1). In addition to this osteogenic expression pattern, hierarchical clustering of differentially expressed genes (DEGs) showed a cluster of upregulated genes in male NCSCs comprising amongst others the known autosomal osteogenic regulator Cytochrome P450 Family 7 Subfamily B Member 1 (CYP7B1) and the lncRNA family with sequence similarity 157 (FAM157C) (Fig. 3A-B). Amongst other, this cluster further included Y-linked genes like the lysin demethylase 5D (KDM5D) and the putative osteogenic regulator Ubiquitin Specific Peptidase 9 Y-Linked (USP9Y) (Fig. 3A-B). Contrarily, female NCSCs revealed an increased expression of BMP7 (table 1) as well as a cluster of upregulated genes comprising the pro-osteogenic regulators KDM6A and XIST compared to male after 30 days of differentiation (Fig. 3C-D). However, we observed negative regulators of osteogenic differentiation like the X-linked gene taxilin gamma (TXLNG) (Fig. 3C-D) or MIR137HG (table 1) to be significantly higher expressed in female NCSCs in comparison to their male counterparts.

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Figure 3. Hierarchical clustering of differentially expressed genes reveal a sexually dimorphic osteogenic regulator profile between male and female NCSCs undergoing differentiation. (A-B) Cluster of upregulated genes in male NCSCs comprising autosomal osteogenic regulator CYP7B1 as well as the Y-linked genes KDM5D and USP9Y. (C-D). Female NCSCs showed cluster of upregulated genes including the pro-osteogenic regulators KDM6A and XIST as well as the negative regulator TXLNG.

Table 1: Differentially expressed pro- and anti-osteogenic genes not included in clusters (Figure 3) between female and male NCSCs after 30 days of differentiation.

Gene name	ENS ID	Down-/upregulation in female NCSCs compared to male NCSCs (log2 fold change)	P-value
BMP4	ENSG00000125378	-1.2	0.014
MSX2	ENSG00000120149	-0.8	0.013
COL11A1	ENSG00000060718	-2.8	0.015
COL4A4	ENSG00000081052	-1.2	0.043
ITGA1	ENSG00000213949	-0.8	0.021
FOS	ENSG00000170345	-1.5	0.000
BMP7	ENSG00000101144	2.6	0.008
MIR137HG	ENSG00000225206	1.0	0.037

In addition to the determination of distinct clusters of differentially expressed genes or single osteogenic markers and regulators, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) term analysis. In accordance to our findings showing differences in the osteogenic differentiation between male and female NCSCs, differentially expressed genes belonged amongst others to the osteogenic-related KEGG pathways “ECM-receptor-interaction”, “cell adhesion molecules”, “focal adhesion”, “TGF-beta signaling pathway” or “MAPK signaling pathway” (Fig. 4C). Accordingly, differentially expressed genes were annotated to the GO terms “cell adhesion”, “cytoskeleton organization” or “extracellular region” (Fig. 4C).

Figure 4. (A-B) Ribosomal Protein S4 Y-Linked 1 (RPS4Y1) and KDM5D (depicted in cyan) were most significantly downregulated in female NCSCs after 14 days and 30 of differentiation compared their male counterparts. (C) Differentially expressed genes between male and female NCSCs belonged amongst others to the osteogenic-related KEGG pathways and GO terms (depicted in orange).

Identification of Y-linked lysin demethylase 5D as a novel regulator of osteogenic differentiation in male NCSCs

Among the genes in cluster A (Fig. 3A-B) upregulated in male NCSCs undergoing osteogenic differentiation, we found Ribosomal Protein S4 Y-Linked 1 (RPS4Y1) and lysin demethylase 5D (KDM5D) to be most significantly and strongly downregulated in female NCSCs after 14 days of differentiation (Fig 4A). In addition to DEAD-Box Helicase 3 Y-Linked (DDX3Y), the transcripts of RPS4Y1 and KDM5D were likewise most significantly downregulated in female NCSCs undergoing osteogenic differentiation for 30 days (Fig 4B). While increased transcription of RPS4Y1 might be a potential hint of differences in protein translation, histone methylation and histone demethylases like KDM4A are discussed to regulate osteogenic differentiation (reviewed in (20))(21). Thus, we particularly focused on investigating the regulatory functions of KDM5D in more detail. We used siRNAs against five distinct loci in the mRNA of KDM5D (Fig. 5A) followed by their lentiviral transduction into male NCSCs. Firstly, knockdown of KDM5D was verified by qPCR depicting a significantly reduced expression of KDM5D compared to untreated control and GFP-transduced control in undifferentiated NCSCs (Fig. 5B). Secondly, knockdown of KDM5D resulted in a strong inhibition of osteogenic differentiation of male NCSCs shown by reduced Alizarin Red S-stained calcium deposition after 30 days of culture on collagen type I fibers (Fig. 5C). Quantification of Alizarin Red S-stainings confirmed the significant inhibition of male osteogenic differentiation upon siRNA-mediated knockdown of KDM5D (Fig. 5D-E). In addition, we applied KDOAM-25 for pharmacological inhibition of KDM5D in male NCSCs. After 30 days of culture on collagen type I fibers, we observed a significant reduction of calcium deposition in male NCSCs with increasing concentrations of KDOAM-25 (1 μ M – 100 μ M) compared to untreated control (Figure 6A-C). These findings provide evidence of KDM5D being a novel crucial regulator of osteogenic differentiation in male craniofacial NCSCs.

Figure 5. Knockdown of KDM5 significantly impairs the osteogenic differentiation capacity of male NCSCs. (A) Schematic view on siRNA design. (C) qPCR analysis validating the knockdown of KDM5D. (C-E) Knockdown of KDM5D strongly inhibited the capability of male NCSCs for depositing calcium after 30 days of culture on collagen type I fibers compared to untreated and GFP-transduced cells. (D-E) Quantification of Alizarin Red S confirmed the significant inhibition of osteogenic differentiation in male NCSCs after knockdown of KDM5D. Mann Whitney test, *P < 0.05 was considered significant.

Figure 6. Pharmacological inhibition of KDM5D significantly reduces the capability of male NCSCs to undergo osteogenic differentiation. (A-B) Exposure of male NCSCs to increasing concentrations (1µM – 100 µM) of KDOAM-25 resulted in a significant reduction of calcium deposition after 30 days of differentiation compared to untreated control. (C) Quantification of Alizarin Red S confirmed the significant inhibition of male osteogenic differentiation upon inhibition of KDM5D by KDOAM-25-treatment. Mann Whitney test, *P < 0.05 was considered significant. n.d.: not detectable.

Discussion

In the present study, we demonstrate a decreased capacity of calvarial bone recovery in female rats as well as a cell autonomous delay in osteogenic differentiation of female human craniofacial stem cells compared to their male counterparts. Our findings particularly reveal a significantly decreased bone volume and bone mineral density in female rats compared to male animals upon transplantation of Collagen fibers or Spongostan into critical-size calvarial defects. Accordingly, we previously reported the highly efficient closure of the calvarial lesions in male rats by Collagen fibers or Spongostan (18) even in comparison to other clinically approved bone substitute materials like NanoBone or Actifuse (19). In line with our present observations, Meszaros and coworkers reported a significantly increased regeneration of cranial bone defects in male mice compared to their female counterparts after transplantation of gelatin sponges containing muscle-derived stem cells (12).

Transferring these promising findings to the human system, we further observed a strong delay in osteogenic differentiation of adult human craniofacial neural crest-derived stem cells from female donors compared to those isolated from male ones. This observation is in accordance with the reduced capacity of female individuals for bone regeneration (1), reviewed in (2, 3, 4) and increased risk of fractures developing nonunion (1, 6) and elevated prevalence of osteoporosis (7-9). Although certain osteogenic markers like Runx2 or Osteonectin were not differentially expressed between male and female NCSCs, global transcriptional profiling revealed a broad range of differentially expressed genes between male and female NCSCs after 30 days of differentiation. Amongst others, these genes belonged to the KEGG pathway “ECM-receptor-interaction”, “cell adhesion molecules”, “focal adhesion”, “TGF-beta signaling pathway”, and “MAPK signaling pathway” or the GO term “cell adhesion”. In line with our present findings, KEGG pathways like “focal adhesion”, “ECM-receptor interaction” and the “MAPK signaling pathway” were previously reported to be enriched in human adult stem cells undergoing osteogenic differentiation compared to undifferentiated controls (Shang et al. 2016). Particularly the pathway “ECM-receptor interaction” and expression of the gene ITGA1 can be directly linked to an interaction of NCSCs with the substrate consisting of collagen type I fibers harboring a nanoporous surface of 30 nm pores, in turn guiding their osteogenic differentiation (16).

Next to characteristic osteogenic markers like BMP4 (22, 23), MSX2 (24), COL4A4 and COL11A1 (25), we observed a range of osteogenic regulators strongly upregulated in male NCSCs after osteogenic differentiation compared to their female counterparts. Notably, we found autosomal genes like C-FOS (chromosome 14), CYP7B1 (Cytochrome P450 Family 7 Subfamily B Member 1, chromosome 16) and FAM157C (family with sequence similarity 157, chromosome 8) among these regulators. Although C-FOS was described to determine the differentiation of murine hematopoietic cells into osteoclasts (26), it was likewise shown to precede osteogenic differentiation of cartilage cells (27). Likewise, Ohta and

colleagues reported fracture healing to be accompanied by an induction of C-FOS expression in osteoblasts within the ossifying callus (28). Next to C-FOS, our present data revealed an elevated expression of CYP7B1 in male NCSCs after 30 days of osteogenic differentiation. CYP7B1 encodes a P450 enzyme, namely the oxysterol 7 α -hydroxylase, which metabolizes the cholesterol metabolite 27-hydroxycholesterol (27HC), the first identified endogenous selective estrogen receptor modulator (reviewed in (29)). In patients, CYP7B1 mutations consequently lead to an accumulation of circulating 27HC (30), which was in turn reported to inhibit the effects of estrogen (31). Notably, CYP7B1 can be also considered as a major regulator of osteogenesis, as DuSell and coworkers demonstrated a KO of Cyp7b1 in female mice to result in decreased bone mineral density and bone formation rate compared to wildtype controls (32). Likewise, we detected a male-specific elevated expression of the lncRNA FAM157C, which is commonly known to be expressed in the human bone marrow (33). Li and coworkers showed deletion of FAM157C as one rare copy number variant being present in female patients suffering from turner syndrome (34). Turner syndrome is a congenital disease caused by loss of one X-chromosome, which results amongst other phenotypic characteristics in a severe bone fragility including low bone mineral density and an elevated fracture risk (reviewed in (35)). In this line, the present observations reveal FAM157C as well as CYP7B1 and C-FOS as autosomal regulatory hallmarks of the osteogenic differentiation of male adult human stem cells compared to their female counterparts. Our findings may suggest the reduced expression of these regulators in differentiating female NCSCs as one underlying mechanism for the delay in osteogenic differentiation observed here. Next to autosomal osteogenic regulators, the expression of genes like USP9Y located on the Y-chromosome was significantly increased in male NCSCs compared to female NCSCs undergoing osteogenic differentiation. Interestingly, Shang and coworkers reported USP9Y to be alternatively spliced upon osteogenic differentiation (36), suggesting a regulatory role in osteogenic differentiation. We further particularly focused on one member of the Y-linked Lysin demethylase (KDM) family, namely KDM5D, which was highly upregulated in male NCSCs after 30 days of osteogenic differentiation compared to female NCSCs. Histone methylation is commonly known to be crucially involved in osteogenic differentiation of stem cells, although the methylation status can either lead to suppression or promotion of osteogenic differentiation (reviewed in (20)). For instance, overexpression of KDM4A specific for H3K9me3 (trimethylated Lysin-9 at histone H3) enabled osteoblast differentiation of mesenchymal stem cells (21). Likewise, Yin and coworkers demonstrated the histone H3K4 trimethyltransferase Ash1l (absent, small, or homeotic-like) to promote osteogenic differentiation and osteogenesis (37). On the contrary, KDM7A and KDM5A were reported to negatively regulate osteogenic differentiation of mesenchymal progenitor cells (KDM7A) or human adipose-derived stem cells (KDM5A) (38, 39). To the best of our knowledge, our present findings identify for the first time KDM5D, which demethylates H3K4me2/H3K4me1, as a critical driver of male

osteogenic differentiation. Next to its elevated expression in male NCSCs after osteogenic differentiation, si-RNA-mediated knockdown or pharmacological inhibition of KDM5D significantly reduced the osteogenic differentiation capacity of male NCSCs. Therefore, our findings demonstrate the expression of KDM5D as prerequisite for the faster osteogenic differentiation of male NCSCs in comparison to NCSCs from female individuals. In accordance to our findings, Shang and coworkers reported KDM5D to be alternatively spliced in human cartilage endplate-derived stem cells after osteogenic differentiation compared to control (Shang et al. 2016).

While certain osteogenic regulators like KDM5D, C-FOS, CYP7B1 and FAM157C were highly upregulated in male NCSCs after osteogenic differentiation, we observed a completely different expression pattern of osteogenic regulators in female NCSCs after the same exposure period to differentiation stimuli. Here, we found elevated transcript levels of X-linked pro-osteogenic genes like KDM6A or XIST to be upregulated in female NCSCs compared to male ones. Hemming and coworkers reported the lysin demethylase KDM6A to promote osteogenic differentiation of MSCs *in vitro* and *in vivo* (40). The long non-coding RNA (lncRNA) XIST was likewise shown to participate in osteogenic differentiation of NC-derived periodontal ligament stem cells (41). In line with the unchanged expression of osteogenic markers like Osteonectin and Runx2 between male and female NCSCs, these findings indicate the at least partial osteogenic differentiation of female NCSCs after 30 days of cultivation on collagen type I fibers. However, we also observed negative regulators of osteogenic differentiation to be significantly higher expressed in female NCSCs. These regulators particularly included the autosomally located gene MIR137HG (chromosome 1) as well as the X-linked gene TXLNG. Knockdown of MIR137HG was reported to promote the osteogenic differentiation of human adipose-derived stem cells (42). The TXLNG gene encodes FIAT (factor inhibiting activating transcription factor 4 (ATF4)-mediated transcription), which overexpression in mice was shown to lead to a reduced mineral deposition rate in turn resulting in decreased bone mineral density and bone volume (43). These observations are in line with our present observations linking an elevated expression of TXLNG to a delayed osteogenic differentiation and calcium deposition in female NCSCs. The expression of these negative osteogenic regulators in female NCSCs in combination with the decreased expression of major positive regulators like KDM5D, C-FOS or CYP7B1 compared to their male counterparts may build the molecular basis of the observed delay in osteogenic differentiation in female craniofacial NCSCs.

In summary, our present findings show a decreased calvarial bone regeneration in female rats as well as a delayed osteogenic differentiation of female adult stem cells from the craniofacial region compared to their male counterparts. On molecular level, this delay in osteogenic differentiation was accompanied by sex-specific expression pattern of negative osteogenic regulators like MIR137HG and TXLNG in female NCSCs, while male NCSCs revealed an elevated expression of pro-osteogenic

regulators including C-FOS and CYP7B1. We further demonstrate the expression of Y-linked KDM5D as a prerequisite for the faster osteogenic differentiation of male NCSCs in comparison to NCSCs from female individuals. The present observation highlight the relevance of sexual dimorphisms in stem cell behavior for guiding bone recovery in clinical settings.

Materials and Methods

Animals

This study was approved by the responsible authority (LANUV, Approval Ref. No. 81-0204.2018.A188). All animal work was performed in accordance with the policies and procedures established by the Animal Welfare act, the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the National Animal Welfare Guidelines. For this study, a minimum number of 5 male and 5 female white Wistar rats (average weight of 300 g at day of surgery) from our own breeding at the University of Bielefeld were randomly assigned to either group A (empty control, n=5), group B (collagen fibers, n=5), group C (Spongostan, n=5). All animals were housed 2-per-cage under a 12h light/dark cycle with food and water provided *ad libitum*. All efforts were made to minimize potential suffering in accordance to the 3R (replacement, reduction, refinement) guidelines.

Animal Surgery and Postoperative Care

Male rats were anesthetized as previously described (18). Female rats were administered an intraperitoneal injection of “Hellabrunner Mischung” comprising ketamine hydrochloride/xylazine hydrochloride solution (1ml/kg, Sigma Aldrich, Taufkirchen, Germany). The surgical procedure followed the description of Spicer and colleagues (44). Briefly, the fur was shaved and the area of surgery was cleaned using an iodine swab. A skin incision, followed by periosteum removal, was made. Afterwards, on both sides the trepanation was performed using a 5 mm hollow drillhead (Trephines 229 RAL 040: Hager and Meisinger GmbH, Neuss, Germany) and a surgical dental drill (Implantmed: W&H Dentalwerk Bürmoos GmbH, Bürmoos, Austria) with a constant speed of 2000 rpm. The circular bone fragment was lifted out, the defect was washed with sterile saline solution and the holes were treated according to the group the animal was assigned to. For void filling, Spongostan (Ferrosan Medical Devices, Søborg, Denmark; marketed by Ethicon Biosurgery, Johnson and Johnson, New Brunswick, NJ, United States) was commercially purchased, cut into cubic (1 mm³) and pre-wetted in 0.9% NaCl solution (B. Braun Melsungen AG, Melsungen, Germany) for 30 min prior to transplantation (18). Collagen type I fibers were prepared as described previously (16). Thereafter, the periosteum was closed with sutures (Prolene 5-0, Ethicon, Johnson and Johnson, New Brunswick, NJ, USA) and the skin was closed by four single back-and-forth sutures (Prolene 3-0, Ethicon, Johnson and Johnson, New Brunswick, NJ, USA). After surgery, the rats were placed in a warmed incubator and housed singly for 3 days.

Euthanasia and Sample Extraction

Animals were euthanized thirty days postoperatively using the Exposure Line carbon dioxide box bioscope (Ehret, Freiburg, Germany). Calvaria were extracted by removing soft tissue and surrounding bone, followed by fixation using 4% paraformaldehyde (PFA) and storage at 4°C.

Micro-CT

All samples underwent micro-computed tomography as previously described (18) using a SkyScan 1176 (Bruker, Kontich, Belgium).

Histology

Extracted calvaria were fixed with 4% paraformaldehyde (PFA), subsequently decalcified using ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin. Serial sections were stained with Goldner's trichrome stain, while nuclei were stained with hematein followed by microscopical examination.

Osteogenic Differentiation of Human Neural Crest Derived Inferior Turbinate Stem Cells

Human inferior turbinates were obtained during routine surgery after an informed consent process, according to local and international guidelines (Bezirksregierung Detmold, Germany) and NCSCs (inferior turbinate stem cells, ITSCs) were isolated and cultivated as previously described (45). All experimental procedures were ethically approved by the ethics board of the medical faculty of the University of Münster (No. 2012–015-f-S). Osteogenic differentiation was induced topologically using collagen fibers according to our previously reported protocol (16). Briefly, cells were seeded onto collagen fibers endogenously harboring 30nm pores and cultivated in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO₂ in a humidified incubator (Binder, Tuttlingen, Germany). Calcium deposition was visualized by Alizarin Red staining and imaging according to (16) followed by quantification using photometric measurement as shown in (46).

RNA Isolation and Sequencing

For RNA isolation the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) was used according to manufacturer's guidelines. Quality and quantity of the isolated RNA was determined via NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation using the NEB Next Ultra Library Prep Kit (New England Biolabs, Frankfurt am Main, Germany) and sequencing on Illumina Novaseq6000 platform was carried out by Novogene (Cambridge, United Kingdom) followed by bioinformatic

analysis. Hisat2 was used for mapping to the reference genome GRCh38/hg19. Quantification of the gene expression was analyzed via HTSeq and for differential gene expression analysis the DESeq2 R Package was used. Enrichment analysis of differentially expressed coding genes were determined for GO Enrichment via the R Packages GOSep, topGO and hmscan and analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed using KOBAS and PANTHER.

Generation of lentiviral vectors

ShRNAs against KDM5D were commercially purchased (MISSION shRNA Bacterial Glycerol Stock, clone IDs: NM_004653.3-4909s21c1, NM_004653.2-4787s1c1, NM_004653.3-1368s21c1, NM_004653.2-3246s1c1, Sigma Aldrich). Target sequences are depicted in Figure 5A. Lentiviral vectors were generated followed to the protocol of Tang and colleagues (47) using transient cotransfection of HEK 293T cells with a three-plasmid system. Lentiviral transfer vector plasmid, packaging plasmid and envelope plasmid were mixed and 1,25 mM PEI were added and incubated for 10 min at room temperature. Meanwhile, HEK 293T cells were trypsinized, washed twice with 1x PBS and 2×10^6 cells were resuspended in media. The vector/PEI mixture was added to the cells and immediately plated onto a plate and incubated at 37°C and 5% CO₂ in a humidified incubator. After 48h the supernatant containing lentiviral vectors was collected and centrifugated at 3000rpm for 5 min at room temperature followed by filter through a 0,45µm mesh. Virus was transferred into conical tubes and spin at 20000rpm for 2 hours at 4°C in (Beckman TST2838) swinging bucket rotor. After spin the supernatant was discarded and the virus resuspend in a desired volume of serum-free media and stored at -80°C.

siRNA-mediated knockdown and pharmacological inhibition of KDM5D

NCSCs were transduced with lentivirally packaged vectors produced as described above followed by verification of knockdown using qPCR (see below). Knockdown cells were seeded on collagen type I fibers and cultivated for 30 days as described above. Pharmacological inhibition of KDM5D was performed by exposing NCSCs to 1µM, 10µM and 100 µM KDOAM-25 (KDOAM hydrochloride hydrate, SML2774, Sigma Aldrich) for 30 days of osteogenic differentiation on collagen type I fibers as described above.

qPCR

Total RNA was isolated as described above followed by cDNA synthesis using First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). qPCR was performed using Platinum SYBR Green

454 qPCR Super-Mix UDG (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) according to the
455 manufacturers guidelines and assayed with a Rotor Gene 6000 (QIAGEN, Hilden, Germany).

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Competing Financial Interests statement

The authors declare no conflict of interest.

Data availability statement

The RNA-Seq datasets generated and analysed during the current study will be available in the NCBI's Gene Expression Omnibus repository (48) through GEO Series accession number.

Author contributions

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