## Data S1. Supplementary methods and validation of the KDM6A conditional knockout mouse model.

*Examples of mouse genotype identification and grouping basis.* An example of mouse tail clipping PCR genotype identification results is shown in Fig. S1. For lysine demethylase 6A (KDM6A)-Flox, the presence of a 378-bp band indicates a wild-type mouse, a 432-bp band indicates a KDM6A conditional knockout homozygous mouse, and the presence of both bands indicates a KDM6A conditional knockout heterozygous mouse. For R26-CAG-LSL-tdTomato (a Cre-dependent reporter strain in which a loxP-flanked transcriptional stop cassette prevents the expression of the tdTomato fluorescent protein; upon Cre-mediated recombination, tdTomato is expressed, permanently labeling Cre-active cells and their progeny), a 297-bp band indicates a wild-type mouse, a 196-bp band indicates a tdTomato fluorescent protein conditionally activated homozygous mouse, and the presence of both bands indicates a tdTomato fluorescent protein conditionally activated heterozygous mouse. For collagen type II α1 chain (Col2a1)-CreERT2 (expressing CreERT2 specifically in chondrocytes, driven by the Col2a1 promoter), a 415-bp band indicates a wild-type mouse, a 480-bp band indicates a Col2a1-CreERT2 homozygous mouse, and the presence of both bands indicates a heterozygous mouse carrying one Col2a1-CreERT2 allele. Heterozygous and homozygous genotypes are referred to as HE and HO. The genotype of KDM6A $^{flox/flox}$/RosatdTomato/Col2a1-CreERT2 is HO/HE/HE, which is a homozygous mouse with cartilage-specific labeling of tomato red and KDM6A conditional knockout, as shown in sample D272 in the figure. Mice that were heterozygous for all three alleles KDM6A$^{flox/+}$/RosatdTomato/Col2a1-CreERT2 (genotypically represented as HE/HE/HE) were used as controls, as exemplified by sample D287 in the figure.

Western blot analysis of KDM6A protein expression was performed using the chondrocytes from the cartilage callus of the two groups of mice after induction via intraperitoneal tamoxifen injection. The results are shown in Fig. S2. KDM6A expression in the Kdm6a conditional knockout mice was lower than that in the fx/wt group, indicating that the knockout was effective.

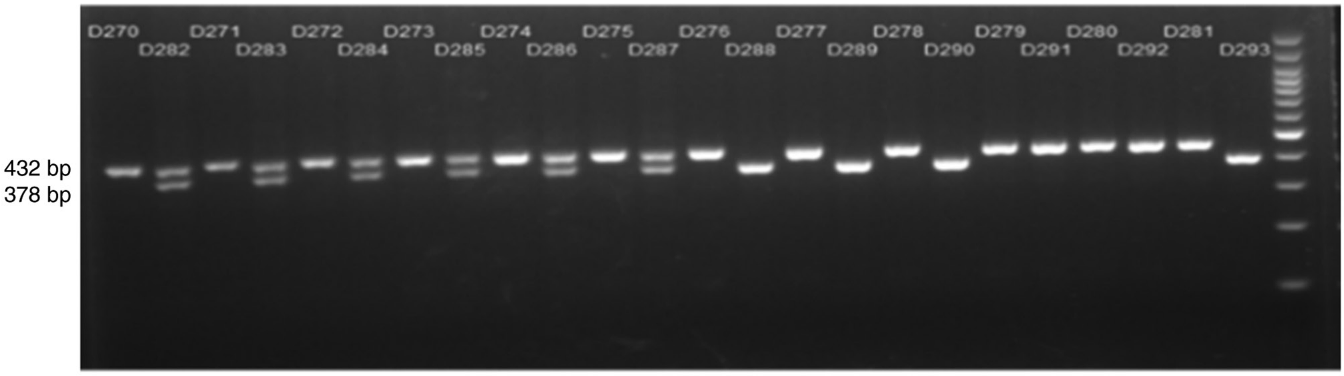
*Effect of KDM6A knockout on the morphological changes of cells during the chondrocyte-osteoblast trans-differentiation process in vitro.* Live cell imaging of the two groups of primary chondrocytes from cartilage callus induced by a normoxic environment to undergo osteoblast trans-differentiation is shown in Fig. S3. Clear red marker signals specific to chondrocytes were observed, and the Col2a1-CreERT2/RosatdTomato model was successfully established. However, through 48-h live cell imaging, only the increase in cytoplasm size and the increase in the fluorescence signal area of the induced trans-differentiated cells were observed, without obvious morphological changes or morphological differences between the two groups of cells.

*Quality control of single-cell sequencing samples.* A single-cell RNA sequencing (scRNA-seq) clustering analysis was conducted on the sample data collected 10 days after fracture. A total of six mouse bone callus samples (including experimental group FX1, FX2 and FX3, and control group WT1, WT2 and WT3) were analyzed, resulting in 86,200 cells. The number of cells in each sample ranged between 11,832 and 16,023, with a median of 1,547-2,073 genes. After filtering, each sample yielded 9,584-13,642 high-quality cells. Using Seurat, cells that died or were under stress were filtered based on several indicators, including intracellular genes, Unique Molecular Identifier (UMI), mitochondrial proportion and removal of multicellularity. The filtering criteria were as follows: i) Number of genes identified in single cells (500-infinity); ii) total number of UMIs in single cells was less than infinity; iii) the expression proportion of mitochondrial genes in single cells was <25%; and iv) multicellularity was removed using the DoubletFinder package. Table SII presents the cell quality control metrics for the 10-days post-fracture samples, including the number of cells before and after filtering, the cell retention rate, and the median number of genes detected per cell for each sample.

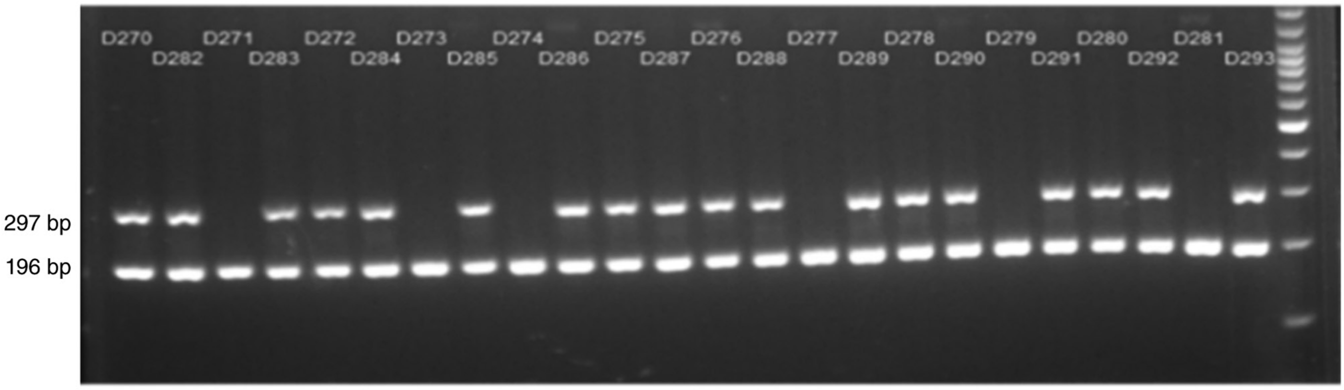
A scRNA-seq clustering analysis was conducted on the sample data collected 21 days after fracture. A total of six bone callus samples from mice (including experimental group FX1, FX2 and FX3, and control group WT1, WT2 and WT3) were processed. Multiple samples were integrated using the anchoring method based on canonical correlation analysis. Through data analysis, a total of 54,586 cells were obtained, with the number of cells in each sample ranging between 7,691 and 10,030, and the median number of genes was 793-1,095. After filtering, 5,364-7,702 high-quality cells were obtained for each sample. Table SIII presents the cell quality control metrics for the 21-days post-fracture samples, including the number of cells before and after filtering, the cell retention rate, and the median number of genes detected per cell for each sample.

Figure S1. Electrophoresis results of tail-cut PCR identification in mice. The labels (such as D270 and D271) represent individual mouse identification numbers. Col2a1, collagen type II α1 chain; KDM6A, lysine demethylase 6A; Mut, mutant; R26, Rosa26 locus; Wt, wild-type.

KDM6A-Flox Mut:432 bp Wt:378 bp



R26-CAG-LSL-tdTomato Mut:196 bp Wt:297 bp



Col2a1-CreERT2 Mut:480 bp Wt::415 bp

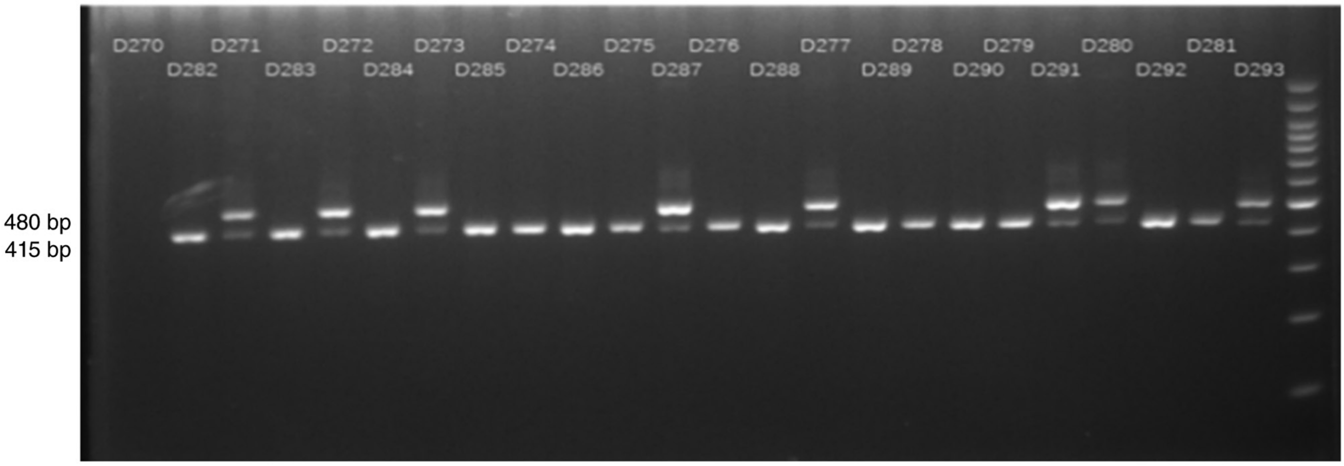


Figure S2. Verification of KDM6A conditional knockout efficiency. fx, Kdm6a conditional knockout mice; KDM6A, lysine demethylase 6A; wt, wild-type.

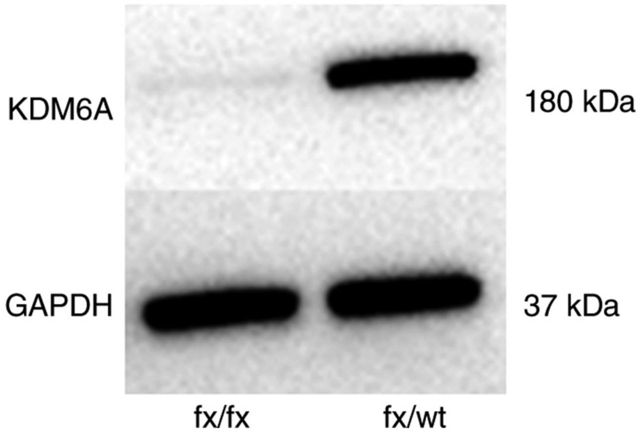


Figure S3. Live-cell imaging of CH-to-osteoblast transdifferentiation over 48 h. Representative phase-contrast/fluorescence images showing the morphological changes and population dynamics of CHs derived from control (fx/wt) and Kdm6a conditional knockout (fx/fx) mice at the initiation (0 h) and after 48 h of osteogenic induction. Scale bar, 200 $\mu$m. CH, chondrocyte; fx, Kdm6a conditional knockout mice; wt, wild-type.

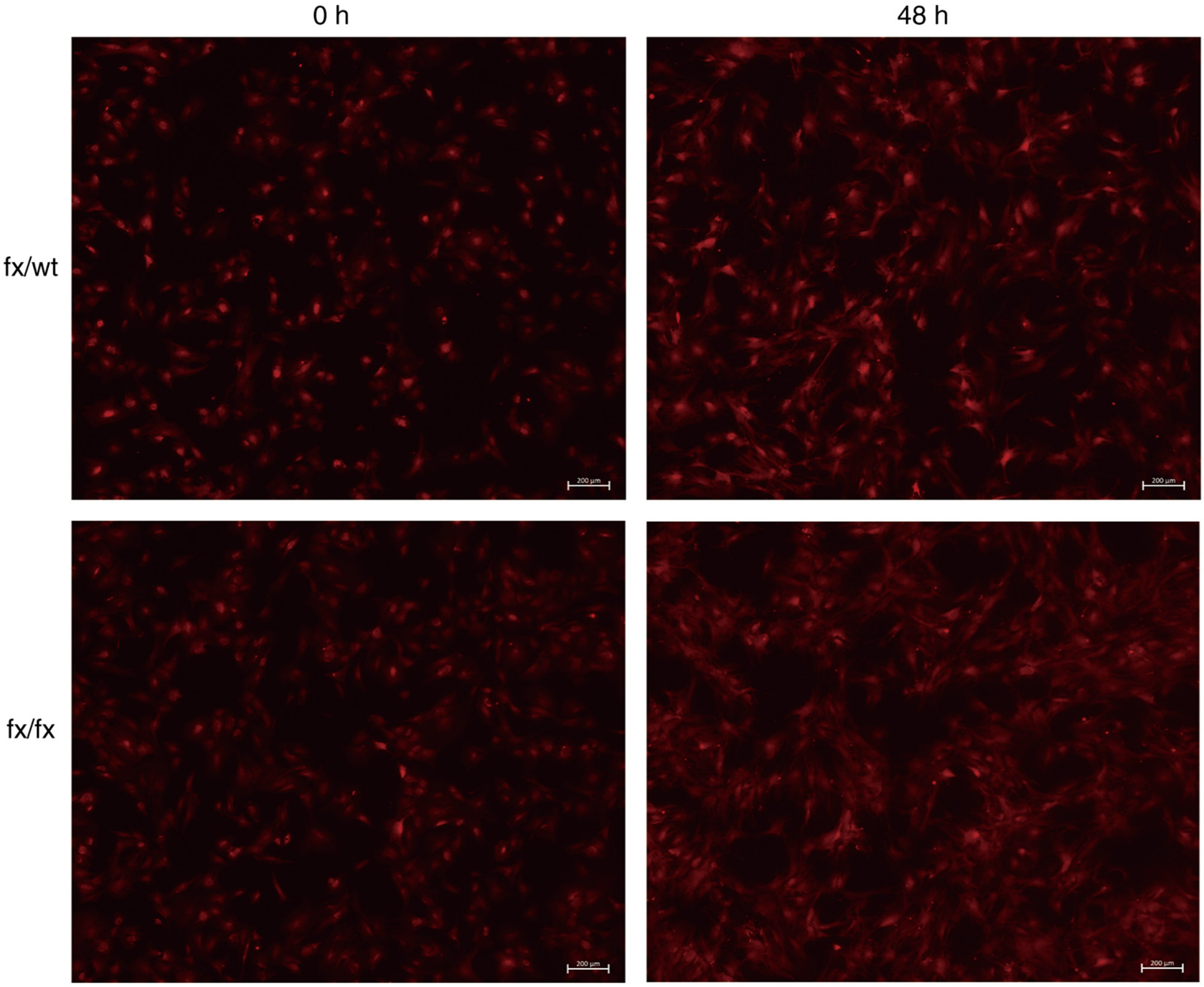


Figure S4. Distribution of basic information of cells in each 10-day sample before and after cell filtering. (A) Distribution of basic information of cells in each sample before filtering. (B) Distribution of basic information of cells in each sample after filtering. The left image shows the distribution of the number of genes detected in individual cells of each sample on the y-axis. The middle image shows the distribution of the total number of UMIs detected in individual cells of each sample on the y-axis. The right image shows the percentage of mitochondrial gene expression per cell (calculated as the proportion of reads or UMI counts mapping to mitochondrial genes out of the total reads/UMI counts per cell) on the y-axis. Cells with poor quality usually have abnormally high mitochondrial gene expression, and mitochondrial gene expression levels can be used as a reference indicator for identifying low-quality cells in the samples. FX, Kdm6a conditional knockout mice; mito, mitochondrial; UMI, Unique Molecular Identifier; WT, wild-type.

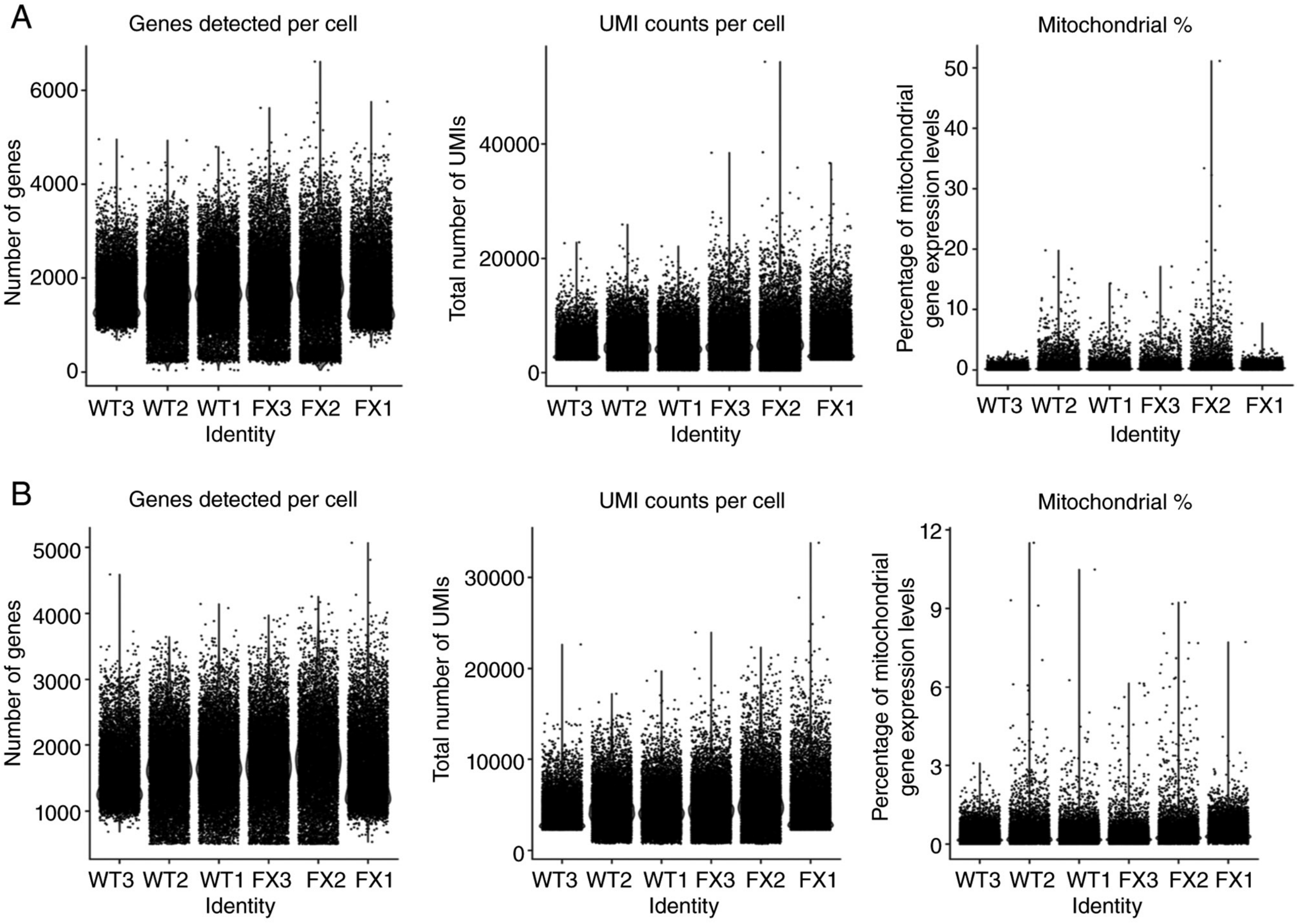


Figure S5. Distribution of basic information of cells in each 21-day sample before and after cell filtering. (A) Distribution of basic information of cells in each sample before filtering. (B) Distribution of basic information of cells in each sample after filtering. The left graph shows the distribution of the number of genes detected in each individual cell of each sample on the y-axis. The middle graph shows the distribution of the total number of UMIs detected in each individual cell of each sample on the y-axis. The right image shows the percentage of mitochondrial gene expression per cell (calculated as the proportion of reads or UMI counts mapping to mitochondrial genes out of the total reads/UMI counts per cell) on the y-axis. Cells with poor quality usually have abnormally high mitochondrial gene expression, and mitochondrial gene expression can be used as a reference indicator for identifying low-quality cells in the sample. FX, Kdm6a conditional knockout mice; mito, mitochondrial; UMI, Unique Molecular Identifier; WT, wild-type.