Supplemental Information

Joint Development Involves a Continuous Influx of Gdf5-Positive Cells

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Inventory of Supplemental Information

Supplemental Figures

Figure S1 shows the generation and validation of the *Gdf5-CreER* model system (Figure 1) by demonstrating the requirement of tamoxifen to induce reporter activity and by showing that homozygous embryos exhibit a phenotype recapitulating that of *Gdf5*-null mice.

Figure S2 provides magnifications of the double ISH for *Gdf5* and *tdTomato* shown in Figure 2.

Figure S3 supports the argument that an influx of Sox9-positive cells contributes to joint formation (Figure 4) by pulse-chase experiments on *Sox9-CreER* mice crossed with *Rosa26-tdTomato* reporter mice, by double immunofluorescent staining for COL2A1 and SOX9, and by showing a similar gene expression dynamic in the elbow joint as was shown for the knee joint.
Figure S1. Validation of the Gdf5-CreER model (related to Figure 1)

(A) Generation of a new knock-in Gdf5-CreER line. (a) Construct design. (b-d’’) Lineage tracing experiment, using the Rosa26-tdTomato mouse as a reporter, demonstrates the effectiveness and accuracy of the newly developed Gdf5-CreER mouse line. Following tamoxifen administration at E13.5, whole-mount preparations from E18.5 embryos exhibit staining in the joints of the vertebrae (b) as well as in various joints of the forelimb (c-c’’) and hindlimb (d-d’’). Magnifications of the forelimb elbow (c’) and autopod (c’’) joints and of the hindlimb knee (d’) and autopod (d’’) joints are shown.

(B) Lineage tracing analysis using Gdf5-CreER mouse crossed with Rosa26-tdTomato mouse as a reporter without tamoxifen administration. Examination at E18.5 shows no tdTomato-positive cells in the elbow (a) and knee (b-c) joints.

(C) Homozygous Gdf5-CreER mice recapitulate the Gdf5-null phenotype. (a,b) Skeletal preparations of forelimb (upper panel) and hindlimb (lower panel) from 3 weeks old control (a, Co) and Gdf5-CreER homozygous (b, Mut) mice show malformed autopods, malformed fibula and a tibia with an increased bend in the mutant. (c-d’) Safranin O staining of sections through the knee joint shows the absence of cruciate ligaments in mutant mice (d) as compared to control mice (c). (c’,d’): Magnifications of the boxed areas in c,d, respectively.

(e,e’): Lineage tracing analysis using Gdf5-CreER homozygous mutant crossed with Rosa26-tdTomato mouse as a reporter demonstrates the expansion of the Gdf5 expression domain following multiple tamoxifen (Tm) administrations (at E11.5, E13.5 and E15.5). Examination at E18.5 showed tdTomato-positive cells occupying a greater area of the autopod, especially in the digits (white arrow). (e’): Magnification of the digit area indicated by an arrow in e. Scale bars represent 1 mm in A and 100 μm in B-C.
Figure S2. Coexpression of Gdf5 and tdTomato in the interzone (related to Figure 2)

(A-C) Double in situ hybridization for Gdf5 (green) and tdTomato (red) genes at E12.5-E14.5, following administration of a single tamoxifen dose at E11.5-E13.5, respectively, shows high overlap between Gdf5- and tdTomato-expressing populations. Magnifications of the boxed areas are shown on the right. Scale bar represents 100 μm.
Figure S3. Joint-forming cells originate from Sox9-positive cells (related to Figure 4)

(A) Pulse-chase lineage tracing experiment using Sox9-CreER mice crossed with Rosa26-tdTomato reporter mice shows that following a single tamoxifen administration at E10.5, all joint lineages are derived from early Sox9-expressing cells. (a,b) Sections through E15.5 knee. (c,d) Sections through E15.5 elbow joint. (e) Double in situ hybridization for tdTomato and Gdf5. (B) Immunofluorescent staining for SOX9 and COL2A1 on sections through E12.5-E15.5 knee (a) and elbow (b) joints. (C) Gdf5 and Sox9 double ISH and COL2A1 immunofluorescent staining on sections through E12-E15.5 elbow (a-f) joints. Arrows indicate a population of Sox9-positive Gdf5, COL2A1-negative cells outside (arrows) and inside (dashed arrows) the interzone. (D) Analysis of Gdf5 gene expression (green) and double immunofluorescent staining for COL2A1 (blue) and SOX9 (red) on section through the knee joint at E14.5. (a) In wild-type mice, an intra-articular population of SOX9-positive, Gdf5, COL2A1-negative cells is indicated by an arrow. (b) Pulse-chase experiment using Gdf5-CreER mice crossed with Rosa26-tdTomato reporter mice. Following tamoxifen (Tm) administration at E11.5, the area corresponding to the intra-articular SOX9-positive, Gdf5, COL2A1-negative population (arrow) is occupied by cells expressing tdTomato but not Gdf5. Scale bar represents 100 μm.
Supplemental Experimental Procedures

Animals

To generate Gdf5-CreER knock-in mouse, a targeting vector (donor construct) was designed to introduce Cre-ER\textsuperscript{T2} fusion gene and an FRT-flanked neo cassette at the ATG of the Gdf5 locus. The construct was electroporated into embryonic stem (ES) cells. PGK-diphtheria toxin cassette was used in the construct to ensure survival of correctly targeted clones only, which would lose the diphtheria toxin due to correct homologous recombination event. ES cells were then injected into recipient blastocysts and chimeric mice were bred with flippase transgenic mice to delete the neo cassette and produce mice that were used to generate a Gdf5-CreER-positive colony. In Figure 1A, arrows indicate the location of the forward and reverse primers used to verify correct targeting of the Gdf5 locus.

Sox9-CreER\textsuperscript{T2} mice were previously described (Soeda et al., 2010).

Tamoxifen administration

For lineage tracing analysis, pregnant female Sox9-CreER mice were administered 0.3 mg/g tamoxifen/body weight in corn oil by oral gavage.

Histological analysis

For paraffin sectioning, embryos were fixed overnight in 4% PFA/PBS, dehydrated to 100% EtOH, embedded in paraffin and sectioned at a thickness of 7 μm. Safranin O staining was performed according to the standard protocol.

Immunofluorescent staining

For paraffin section immunofluorescence, embryos were fixed overnight in 4% PFA/PBS, dehydrated to 100% EtOH, embedded in paraffin and sectioned at a thickness of 7 μm. 10 mM sodium citrate (pH 6.0) was used for antigen retrieval. Slides were incubated overnight at 4°C with primary antibody anti-SOX9 (EMD Millipore) 1:150 in blocking solution. Then, slides were subjected to additional digestion by Proteinase K (sigma) and incubated overnight at 4°C with primary antibody anti-COL2 ((DSHB) 1:50) in blocking solution. Following fluorescence in situ hybridization, slides were incubated overnight at 4°C with primary antibody anti-COL2 (1:50) and anti-SOX9 (1:150) in blocking solution.

Skeletal preparations

Cartilage and bones in whole mouse embryos were visualized after staining with Alcian blue and Alizarin red S (Sigma) and clarification of soft tissue with potassium hydroxide (McLeod, 1980).
In situ hybridization

For double ISH: after hybridization, slides were washed, quenched and blocked. Probes were detected by incubation with anti-fluorescein-POD and anti-DIG-POD (Roche; 1:200), followed by Cy3-, Cy2- or Cy5-tyramide labeled fluorescent dyes (according to the instructions of the TSA Plus Fluorescent Systems Kit, Perkin Elmer). *tdTomato* antisense probe was generated using the following primers: Forward, tccacaacaggactacccat, reverse, cgegcatctcctgtggatca.

Co-localization measurements

Masking was performed using the *Gdf5* channel prior to automatic thresholding (Costes method) for both channels. For *Gdf5* and *tdTomato* co-localization, the value of “% of material *Gdf5* above threshold colocalized” was used. For the prevalence of *tdTomato*-positive, *Gdf5*-negative cells, the value “100%-% of material *tdTomato* above threshold colocalized” was used.

Supplemental References

McLeod, M.J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. Teratology 22, 299-301.