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*BRIEF REPORT*

# Specification of Sprouty2 functions in osteogenesis in *in vivo* context

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**ABSTRACT.** Sprouty proteins are modulators of the MAPK/ERK pathway. Amongst these, Sprouty2 (SPRY2) has been investigated as a possible factor that takes part in the initial phases of osteogenesis. However, the *in vivo* context has not yet been investigated and the underlying mechanisms taking place *in vitro* remain unknown. Therefore, in this study, the impact of *Spry2* deficiency was examined in the developing tibias of *Spry2* deficient (-/-) mouse. The investigation was performed when the osteogenic zone became clearly visible and when all three basic bone cells types were present. The main markers of osteoblasts, osteocytes and osteoclasts were evaluated by immunohistochemistry and RT-PCR. RT-PCR showed that the expression of *Sost* was 3.5 times higher in *Spry2-/-* than in the wild-type bone, which pointed to a still unknown

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mechanism of action of SPRY2 on the differentiation of osteocytes. The up-regulation of *Sost* was independent of *Hif-1α* expression and could not be related to its positive regulator, *Runx2*, since none of these factors showed an increased expression in the bone of *Spry2-/-* mice. Regarding the RANK/RANKL/OPG pathway, the *Spry2-/-* showed an increased expression of *Rank*, but no significant change in the expression of *Rankl* and *Opg*. Thanks to these results, the impact of *Spry2* deletion is shown for the first time in the developing bone as a complex organ including, particularly, an effect on osteoblasts (*Runx2*) and osteocytes (*Sost*). This might explain the previously reported decrease in bone formation in postnatal *Spry2-/-* mice.

**KEYWORDS.** Endochondral bone development, growth plate, mouse, ossification, SPROUTY2

## INTRODUCTION

*Sprouty (Spry)* genes encode cytoplasmic membrane-associated proteins. Initially described in *Drosophila*, the SPRY family of proteins is highly conserved. Four mammalian orthologs (SPRY1-4) exist, which modulate receptor tyrosine kinase (RTK) signaling pathways, although variations of their expression were observed, depending on cell types.[1,2]

Transgenic mice lacking SPRY and SPRED (Sprouty-related, EVH1 domain-containing proteins) family proteins show abnormalities in endochondral (long) bones including a decreased bone growth (achondroplasia-like phenotype) and decreased trabecular bone mass.[3–5] Endochondral bones, unlike the intramembranous ones, involve a chondrogenic developmental step. The developing growth plate may be divided into three compartments: an external fibrous tissue surrounding the periphery, a cartilaginous, and a bony tissue. The cartilaginous part consists of several zones, where chondrocytes differentiate towards the diaphysis: the resting zone, where a pool of less differentiated chondroblasts is localized, proliferating zone, where chondroblasts multiply and further differentiate, and zone of hypertrophy undergoing calcification, degradation and replacement by osseous tissue. The calcifying matrix of cartilage starts to be removed by osteoclasts/chondroclasts in the line of erosion, and the formation of the primary spongiosa involves a cooperation between osteoblasts and osteoclasts in the zone of ossification.[6]

Chondrogenesis in long bones was reported to be impaired in *Spry2* deficient (-/-) mice: chondrocytes undergo increased cell proliferation, but their terminal differentiation is impaired in the femur at embryonic (E) day 18.5.[5] Further, using an *in vitro* approach, *Spry2* overexpression in osteoblastic lineage (MC3T3-E1) resulted in abnormal cell proliferation and differentiation.[7] However, interpretations of the *in vitro* studies were hindered by the fact that proper bone development *in vivo* requires a tight molecular cross-talk networks among osteoblasts, osteocytes and osteoclasts.

To follow the consequences of *Spry2* deficiency *in vivo* on the osteogenic cells (osteoblasts, osteocytes and osteoclasts), the prenatal growth plate within the tibia was examined in the *Spry2-/-* and compared with that from wild-type mice. Attention was focused on initial stages of osteogenesis when the osteogenic zone became apparent in the forming growth plate at prenatal day E18. Since several bone cells interact and play complementary integrated roles in this area, major markers of osteoblasts (early: RUNX2, and late: osteocalcin – BGLAP), osteocytes (sclerostin – SOST) and osteoclasts (receptor activator of nuclear factor κB - RANK) were investigated. To get an overview on the RANK–RANKL–OPG system, RANK and OPG were analyzed simultaneously. All factors were investigated at the level of transcripts (RT-PCR) and proteins (immunohistochemistry).

This investigation, for the first time in an *in vivo* context, aimed to specify the possible impact of *Spry2* deletion on bone-forming cells during endochondral bone development.

## MATERIAL AND METHODS

### Animals

Mice (*Mus musculus*) of the ICR strain were studied at prenatal stage 18. The initial

breeding pairs of the mouse line carrying mutant alleles of *Spry2-/-* were established by Shim et al.[8] and were kindly provided by Dr. Ophir Klein.[9] Mice were maintained in the animal facilities of the Institute of Experimental Medicine, The Czech Academy of Sciences, Prague, Czech Republic. Housing and sacrifice of animals were carried out in strict accordance with the national and international guidelines (ID 39/2009). This study was performed under the supervision of a professional committee for the guarantee of good life-conditions of experimental animals at the Institute of Experimental Medicine, Czech Academy of Sciences, Czech Republic (permit number 81/2017).

The pregnant mice were sacrificed by cervical dislocation and the fetuses were removed at E18. Immediately after removing the embryos, their wet body weights were measured in order to precisely determine their developmental stage.[10]

### *Histology, immunohistochemistry (IHC) and immunofluorescence (IF)*

Mouse hind limbs were fixed in 4% PFA (24 h), decalcified in EDTA, dehydrated in alcohol, treated with xylen and embedded in paraffin. Histological sections (5 μm) were then prepared. Morphological/histological criteria were analyzed after trichrome staining (hematoxylin, alcian blue, sirius red) and TRAP staining, as previously described.[11]

Immunohistochemical detection was performed to show the expression of markers and better specify cell heterogeneity within the developing bone. Antigen retrieval was carried out in citrate buffer (pH = 6.0) for 5 min at 98°C for all markers. The following primary antibodies were applied: SPRY2 (ab50317, abcam, UK), BGLAP (ab93876, abcam, UK), SOST (AF1589, R&D Systems, USA), RANK (ab13918, abcam, UK), RANKL (sc-7628, Santa Cruz Biotechnology, USA), OPG (sc-8468, Santa Cruz Biotechnology, USA), RUNX2 (sc-10758, Santa Cruz Biotechnology, USA). The primary antibodies were diluted 1:50 (RANKL, OPG, RUNX2), 1:100 (SOST, SPRY2), or 1:150 (BGLAP, RANK) and applied overnight at 4°C.

Peroxidase-conjugated streptavidin-biotin system (Vectastain, USA) and chromogen substrate diaminobenzidine (DAB, K3466; Dako, USA) reactions were used to detect the positive cells as brown. Slides were counterstained with hematoxylin. SPRY2 antibody was tested in *Spry2-/-* and wild-type sections (Suppl. 3).

Immunofluorescent analysis was also performed for all the markers listed above. Antigens retrieval and dilutions of antibodies were performed as mentioned for IHC. Then Alexa Fluor® 488 (Thermo Fischer, USA) or Alexa Fluor® 594 (Abcam, UK) was used (1:200) for 40 min at RT. Nuclei were detected by ProLong® Gold Antifade reagent with DAPI (Thermo Fischer, USA).

Sost-positive osteocytes/TRAP-positive osteoclasts were counted in *Spry2-/-* (3/5 animals) and wild-type group (3/5 animals), respectively. Cells were counted in one medial section of tibia for each sample at the same magnification. The number of cells and the total area involved in the analysis were counted/measured using Image J. Results were expressed as total number of cells/total area in each analyzed sample. For the statistical analysis, Mann–Whitney ($p \leq 0.05$) test was applied.

### *Tissue separation*

The region of interest (shown in Suppl. 1) was processed as follows: the tibia (E18) in *Spry2-/-* mice and their wild-type littermates were separated from surrounding soft tissue using a stereoscope. The cartilaginous part was removed and the resting bone part, including the osteogenic part (bony component) of the growth plate and diaphysis, was used for analysis. The separation of fresh tibia was carried out in sterile PBS. One sample for RNA isolation contained two tibias.

### *RT-PCR*

RNA was isolated by RNeasy Mini Kit (Qiagen, USA) and transcribed into cDNA using SuperScript VILO (Invitrogen, USA). RT-

PCR was performed in 10 µl reaction volume containing the one-step master mix gb Ideal PCR Master Mix (Generi Biotech, Czech Republic) using LightCycler 96 (Roche, Switzerland) with preheating at 95°C for 10 min, followed by 40 cycles of 95°C/15 s and 62°C/1 min. The following markers were examined: *Bglap, Sost, Rankl, Rank, Opg, Runx2, Spry2, Hif-1α* (TaqMan Gene Expression Assay, Thermo Fischer Scientific, USA). Expression levels were calculated using the ΔΔCT method, with normalization against *actin* levels (Mouse *Actb*, Mm02619580_g1, TaqMan Gene Expression Assay, Thermo Fischer Scientific, USA). Each group was analyzed in three biological replicates. Reactions were conducted in three replicates per sample for statistical analysis (Student's t-test, $p \leq 0.05$). RT-PCR confirmed that the *Spry2* mRNA, detected in the wild-type tibia, was indeed absent in the *Spry2-/-* samples.

## RESULTS

### The osteogenic part of the tibial growth plate at prenatal day 18 comprises osteoblasts, osteocytes and osteoclasts

The presence of distinct bone cells was demonstrated based on morphological criteria, immunohistochemical detection of specific markers and TRAP-analysis. Osteoblasts showed a cuboidal shape with single round nuclei and were positive for osteocalcin (Fig. 1A). Osteocytes were defined as entrapped in osteocytic lacunae and expressing sclerostin (Fig. 1B). Finally, osteoclasts were multinucleated cells, positive for TRAP staining (Fig. 1C).

### SPRY2 is abundantly expressed in all cell types from the tibia at prenatal day 18

In wild-type mice, intense staining for SPRY2 protein was detected in the osteogenic part and other zones of the growth plate at E18 (Fig. 1D–G): in osteoblasts, osteocytes and even in some osteoclasts (Fig. 1D). SPRY2 was also detected in the periosteum (Fig. 1E) and in the cytoplasm of chondrocytes within the resting, proliferating (Fig. 1F), and hypertrophic zones of the cartilage (Fig. 1G).

### Osteogenic markers are expressed in the growth plate at prenatal day 18

In the osteogenic zone within the wild-type growth plate at E18, osteoblasts abundantly expressed BGLAP (Fig. 1H, $H_1$), RUNX2 (Fig. 1M, $M_1$), and some were even positive for RANKL (Fig. 1K, $K_1$) and OPG (Fig. 1L, $L_1$). Osteocytes were strongly positive for SOST (Fig. 1I, $I_1$), some were also positive for RUNX2 (Fig. 1M, $M_2$) and BGLAP (Fig. 1H, $H_2$). Osteoclasts, and mostly their precursors, were positive for RANK (Fig. 1J, $J_1$).

### Spry2 *deficiency impacts the expression of osteogenic markers*

In order to find out whether *Spry2* deficiency could impact osteogenic pathways in the developing tibia, an RT-PCR-based gene expression was analysis was performed. RT-PCR, performed on the osteogenic part of tibias of the *Spry2-/-* and wild-type mice at E18, revealed statistically significant changes in the expressions of *Sost, Runx2* and *Rank* (Fig. 2B, C, D). The expression of *Sost* mRNA increased by almost 250% ($p = .004674$) (Fig. 2B) in *Spry2-/-* samples, while the expression of *Rank* increased by 80% ($p = .042324$) (Fig. 2D). Conversely, a slight decrease in the expression of *Runx2* was observed in *Spry2-/-* (30%, $p = .011147$) (Fig. 2C). *Spry2-/-* did not show statistically significant changes in the expression of osteocalcin (*Bglap), Rankl, Opg* (Fig. 2A, E, F) and *Hif-1α* (data not shown). These data are available expressed also in –ddCT values for individual animals (Suppl. 2).

The significantly altered expression of mRNA levels was examined also at protein level in the histological sections. While there was a visibly higher number of cells positive for RANK, mostly in peritrabecular regions (Fig. 2J, K), the distribution of SOST-positive cells did not seem to differ when comparing samples from *Spry2-/-* and wild

FIGURE 1. Immunohistochemical/immunofluorescent identification of osteogenic markers and SPRY2 in wild-type tibia at E18.
Localization of osteoblastic cells identified as cuboidal cells with a single nucleus and expressing BGLAP (A), osteocytes entrapped in bone matrix and expressing SOST (B), and osteoclasts identified as large multinuclear cells positive for TRAP staining (C). Detection of SPRY2 in growth plate of tibia (D, E), with focus on resting, proliferating (F) and hypertrophic cartilage together with osteogenic zone (G). BGLAP (H) was localized in osteoblastic ($H_1$), and osteocytic cells ($H_2$), SOST (I) in osteocytes ($I_1$). In growth plate, RANK (J) was detected in osteoclastic cells ($J_1$), RANKL (K) in osteoblastic cells ($K_1$), OPG (L) in osteoblastic cells ($L_1$), and RUNX2 (M) in osteoblastic ($M_1$) and osteocytic cells ($M_2$). HZ (hypertrophic zone of cartilage), OZ (osteogenic zone), PB (periosteal bone), PS (primary spongiosa), PZ (proliferating zone of cartilage), RZ (resting zone of cartilage). For immunofluorescent labeling, positive signal is green, autofluorescent erythrocytes are orange in figures E-I, K-M. Positive signal is red, autofluorescent erythrocytes are green in figure J. Yellow arrowheads point to osteoblasts, green ones to osteocytes, and black ones to osteoclasts. Scale bar = 30 μm (A-C), 100 μm (E-M), 50 μm ($H_1$-$M_1$, $H_2$, $M_2$).

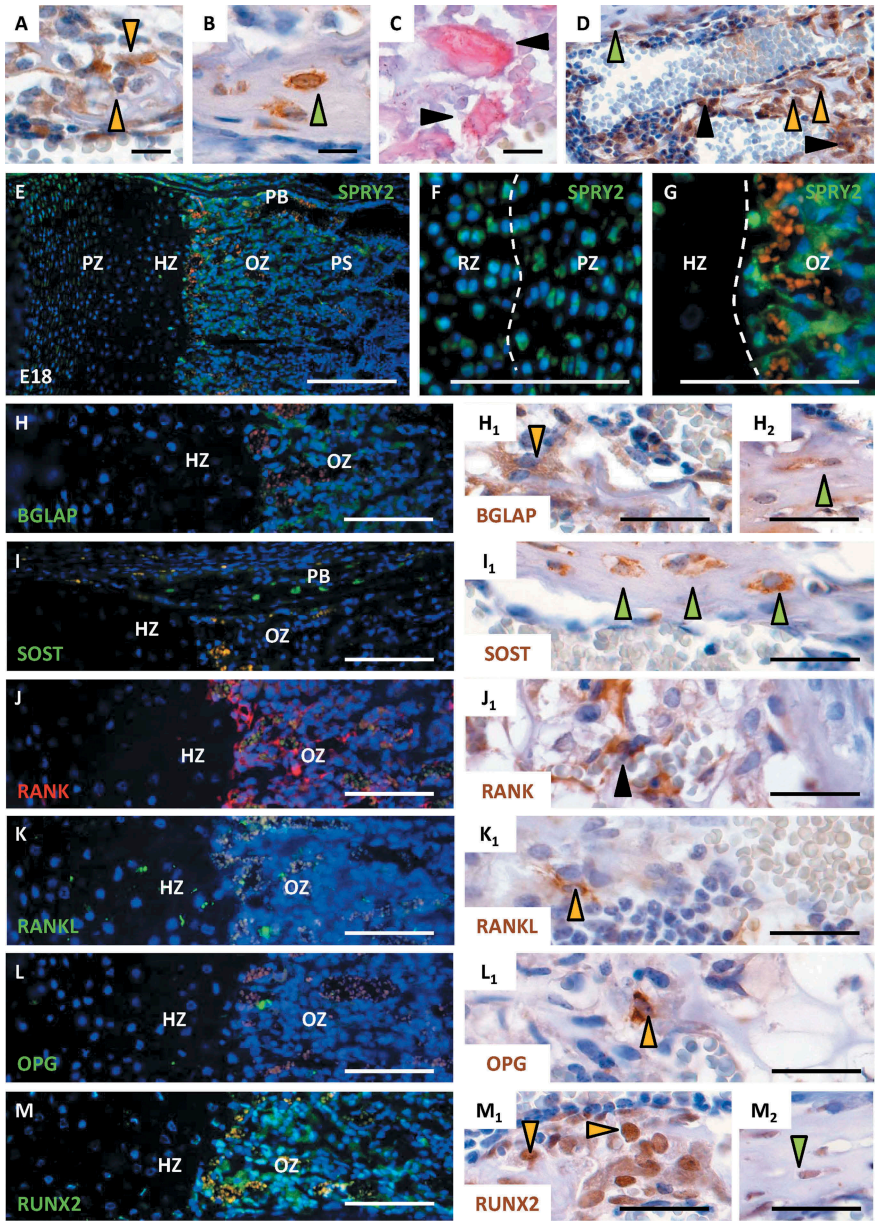
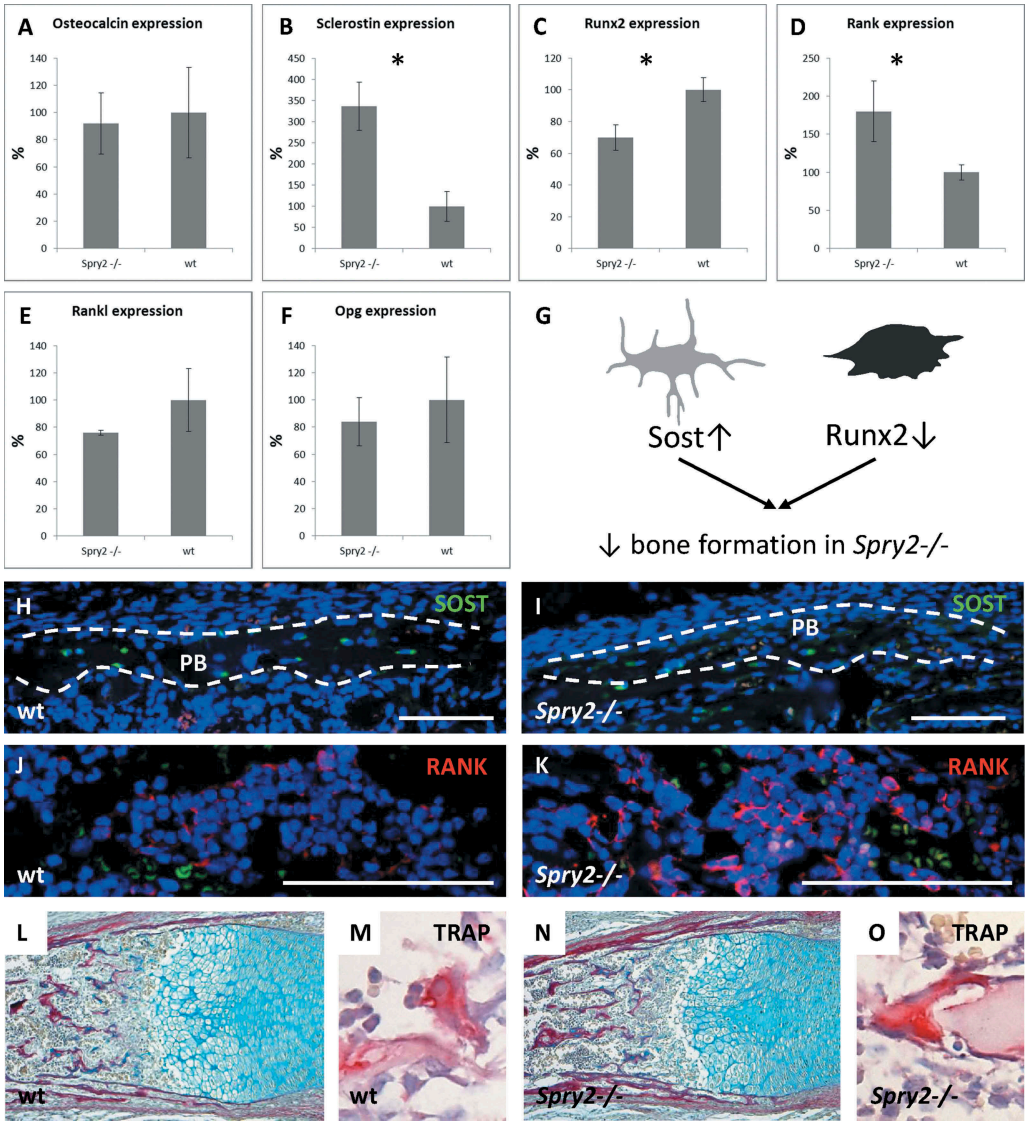


FIGURE 2. Comparison of tibia in *Spry2-/-* and wt at E18
Expression of osteocalcin (A), sclerostin (B), *Runx2* (C), *Rank* (D), *Rankl* (E), *Opg* (F), a tentative scheme of the molecular background of bone phenotype in *Spry2-/-* bones (G). Asterisk, p ≤ .05. Immunofluorescent detection of SOST (H, I) and RANK (J, K) in wild-type and *Spry2-/-* tibias, histology of the growth plate was shown after trichrome staining (L, N) and osteoclastic cells were detected by TRAP assay (M, O). PB (periosteal bone). For immunofluorescent labeling, positive signal is green, autofluorescent erythrocytes are orange in figures H, I. Positive signal is red, autofluorescent erythrocytes are green in figure J, K. Scale bar = 100 μm.




types (Fig. 2H, I). Quantification of SOST-positive cells in immunolabelled samples revealed slightly increased number without statistical significance in *Spry2-/-* mice (Suppl. 3D). The slight decrease

in *Runx2* expression, as detected at mRNA level, was not observed at the protein level (data not shown).

Although the gene expression in *Spry2-/-* was different from that observed in the wild type, there was no obvious consequence at the bone histological appearance in the mutant, when compared to wild type (Fig. 2L, N). The decrease in TRAP-positive osteoclastic cells in *Spry2-/-* mice (Fig. 2M, O) appeared as statistically non-significant (Suppl. 3C).

## DISCUSSION

Bone development involves its formation including extracellular matrix secretion and mineralization (mediated by osteoblasts), and resorption (mediated by osteoclasts). The differentiation of both cell types is linked and their functions are tuned by osteocytes.[12] In endochondral (long) bones, osteogenesis includes a cartilaginous step. Joo et al.[5] have reported alterations in long bone development in *Spry2-/-* mice, which were related to growth plate chondrocytes next to the zone of ossification. Since *Spry2* expression precedes the functional differentiation of osteoblasts, an effect on early osteogenesis was suspected.[13,14] Therefore, we searched for early consequences of SPRY2 deficiency in main bone cell types, osteoblasts, osteocytes and osteoclasts, during the prenatal formation of the growth plate in *Spry2-/-* mice *in vivo*.

The first step was thus to examine SPRY2 expression in cells of interest in wild-type mice. Immunolabelling of the prenatal tibia showed that all these three cell types were positive for SPRY2, within the zone of ossification, as well as some other cells outside of it (e.g. chondrocytes).

Based on immunohistochemistry, osteoblasts, expressing their major marker, BGLAP (osteocalcin), were also positive for RUNX2 in the wild-type bone. RUNX2 is an inducer and an early marker of osteoblast differentiation,[15] which is in agreement with its presence in the early growth plate. Modulation of *Runx2* expression by *SPRY2* was documented also in other system.[14]

Regarding osteocytes identified by their typical marker, sclerostin,[16] *Sost* mRNA was strongly up-regulated in *Spry2-/-* tibias. Immunolabelling showed only a slight non-significant increase of SOST-positive osteocytes in *Spry2-/-* tibias. Therefore, the *Sost* mRNA changes were not due to an altered number of cells. The increased expression of *Sost* was neither mediated by the positive regulator RUNX2.[17] Although RUNX2 was immunodetected in osteocytes, it was shown as decreased in *Spry2-/-* tibias. The increased *Sost* expression was caused neither by an increased HIF-1α signaling, a positive regulator of *SOST* expression,[18–20] since HIF-1α, a downstream molecule of SPRY2,[21] had a similar expression in wild type and *Spry2-/-*. Thus, the regulation of *Sost* remains elusive, although neither connected with Runx2 nor Hif-1α.

The third type of bone-related cells (osteoclasts) was impacted via an increased expression of *RANK* in *Spry2-/-* mice. *RANK* is involved in osteoclastic differentiation from their hematopoietic precursors, through osteoblastic RANK ligand[22,23] showing a decreasing non-significant trend in expression as reported here. This imbalance resulted in only slightly non-significantly decreased number of TRAP-positive osteoclasts in *Spry2-/-* tibias at E18 as observed by Joo et al.[5] Immunolabelling showed that RANK was mostly detected in mononuclear precursors located in intertrabecular regions.

Joo et al.[5] have described *Spry2−/−* pups as indistinguishable from their wild-type littermates in their body weight with a slightly abnormal ratio between proliferating and hypertrophic zones of chondrocytes at E18. Similarly, when compared to the wild type, the histological appearance of *Spry2-/-* tibias did not show differences at E18 in our analysis. However, 6 weeks old *Spry2-/-*mice exhibited a decrease in bone volume/tissue volume, trabecular number and thickness, an increased trabecular spacing and a reduced mineralizing function of osteoblasts.[5] This postnatal phenotype can be supported by our prenatal investigation at molecular level. Although SPRY2 may apparently be involved in osteoclastic

differentiation, the decreased bone formation in *Spry2−/−* mice seems to be caused by abnormal functions of osteoblasts or osteocytes. Since *Runx2* deficient mice show an impaired ossification,[24] decreased expression of *Runx2* may result in decreased osteoblastic differentiation and thus in decreased ossification during development. The same trend can relate to an increased expression of *Sost*. Indeed, SOST acts as a negative regulator of bone metabolism and transgenic mice overexpressing *Sost* show a decrease in bone formation rate, bone volume and bone mineral density.[25,26]

## CONCLUSION

Using the developing long bone as a model organ, the effects of *Spry2* deletion on osteogenic cells appear complex in part because of its ubiquitous expression and also due to the multiple cell-specific effects of the encoded protein. This study showed differential tuning of distinct markers for a given cell type, as well as differences in the pattern of expression from one bone cell type to another. The present *in vivo* study brings complementary data to information gained from studying cell lines *in vitro*, where the associated loss of specific cell-cell interactions (through heterotypic cell contacts or diffusible molecules) may also impair regulatory mechanisms, as they exist in physiological conditions. Based on our observation, the decreased bone formation and mineralization of postnatal *Spry2-/-* mice might be related to the abnormal expression of osteoblastic *Runx2* and osteocytic *Sost*.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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## SUPPLEMENTARY MATERIAL

Supplemental data for this article can be accessed on the publisher's website.

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