RESEARCH REPORTS
Biological

W. Yang[1], M.A. Harris[1], Y. Cui[1], Y. Mishina[3], S.E. Harris[1], and J. Gluhak-Heinrich[2]*

[1]Department of Periodontics and [2]Department of Developmental Dentistry, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA; and [3]Department of Biologic and Material Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI 48109, USA; *corresponding author, gluhak@uthscsa.edu

Bmp2 Is Required for Odontoblast Differentiation and Pulp Vasculogenesis

J Dent Res DOI: 10.1177/0022034511424409

APPENDIX

TISSUE PREPARATION AND *IN SITU* HYBRIDIZATION METHOD AND NUMERICAL ESTIMATION OF LEVEL OF EXPRESSION

This method was used to obtain numerical *in situ* hybridization signal and data for the following papers: Gluhak-Heinrich *et al.* (2008a,b, 2010), Harris *et al.* (2008), Li *et al.* (2008), Robling *et al.* (2008), and Yang *et al.* (2009).

Preparation of Histological Sections

Mouse maxillae were dissected rapidly in the presence of αMEM media and fixed in freshly prepared 4% paraformaldehyde made with RNase-free (DEPC-treated) water, for 48 hrs, 4°C, with rotation/shaking at 30 rpm. The tissue was rinsed with RNase-free PBS and then placed in 15% EDTA, pH 7.5, without fixation, for 6 wks at 4°C; medium was changed 2 times *per* wk. Samples were dehydrated in increasing concentrations of methanol (on ice), embedded in paraffin, and sectioned at 6- to 8-μm thickness.

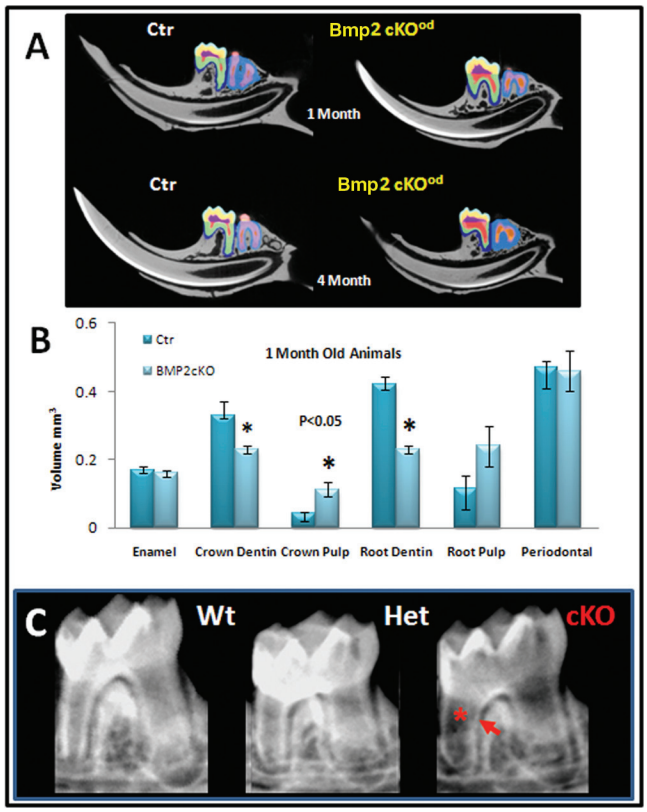
Appendix Table. Ages and Numbers of Animals Used for Various Procedures

Age	Method	Number of Animals
1 day	IHC	6
5 day	IHC	10
5 day	ISH	14
5 day	Histology	8
5 day	X-ray	4
2 wks	Histology	4
2 wks	X-ray	7
1 mo	IHC	12
1 mo	ISH	8
1 mo	Histology	6
1 mo	X-ray	9
1 mo	μCT	6
4 mos	IHC	6
4 mos	ISH	6
4 mos	Histology	6
4 mos	X-ray	39
4 mos	μCT	6
8 mos	X-ray	23

IHC = immunohistochemistry, ISH = *in situ* hybridization.

Preparation of Probes

Mouse RNA probes were used for hybridization, and antisense and sense RNA probes were transcribed with SP_6, T3, and/or T_7 polymerase. All probe sequences from DNA clones were checked for sequence specificity and for any buried repetitive elements. If present or if domain showed homology to other proteins, the probe was recloned without these repetitive sequences. Linearized RNA probes were transcribed *in vitro* in the presence of digoxigenin-UTP-NTP mixture (Roche #11277073910). The probes were checked on a denaturing gel, and, in most cases, one major band with some minor transcripts was shown, depending on the size of the insert. The probe was then hydrolyzed in 40 mM $NaHCO_3$/60 mM Na_2CO_3, pH 10.2, for the desired time at 60ºC. The probes were an average size of 200 to 300 nucleotides, as determined by denaturing gel electrophoresis. Before storage at –80°C, the RNA probe concentrations were estimated by "DOT" blot on a positive charged nylon membrane (Appendix Figure 5). All digoxigenin-labeled probes and control standard digoxigenin-labeled RNA were serially diluted to 1:10, 1:100, 1:1000, and 1:10,000 (it is essential for a control to be run). One μL of each dilution was dropped onto a nylon membrane and crosslinked with a UV transilluminator. First, the filter was washed briefly in Buffer 1 (100 mM Tris-HCl, 150 mM HCl, pH = 7.5), and 5 min in Buffer 2 (buffer 1 + 2% blocking reagent, Roche), then treated with anti-digoxigenin antibody-alkaline phosphatase conjugate (Roche), diluted 1:5000 in Buffer 2. Next, we performed 2 washes for 5 min each in Buffer 1 and 2 min in Buffer 3 (100 mM



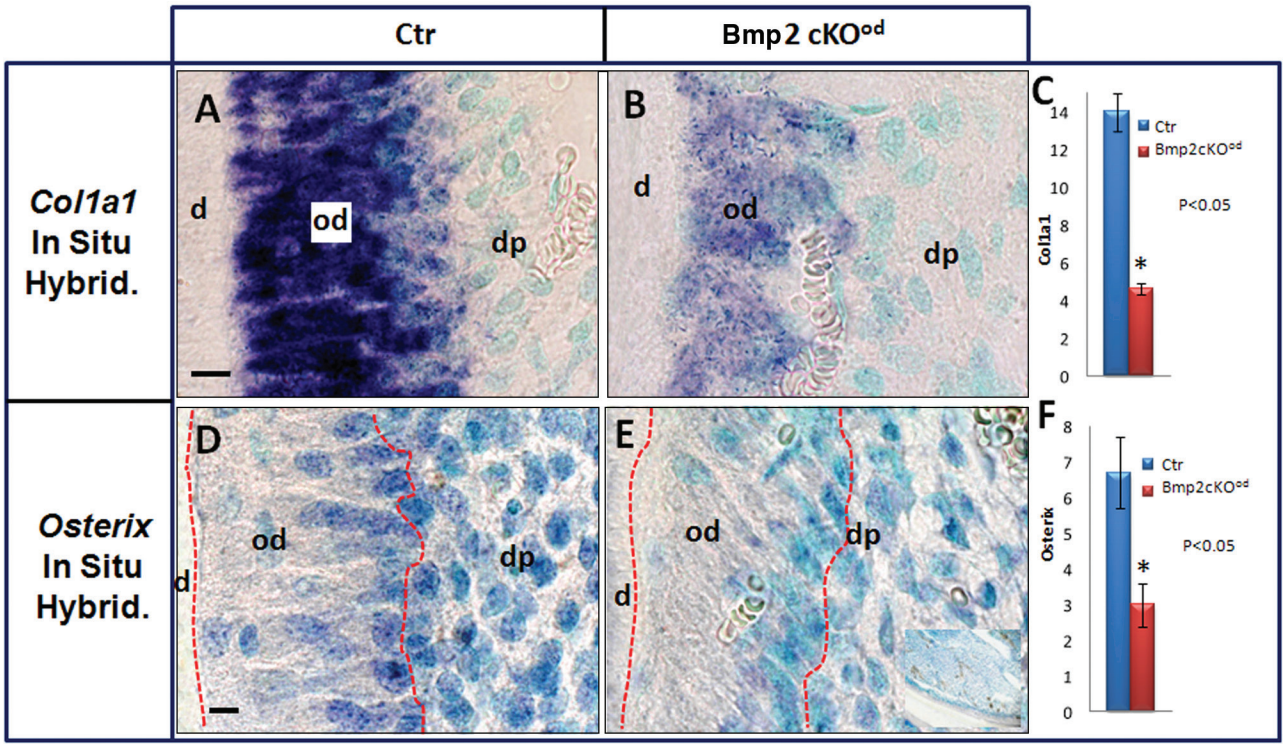
Appendix Figure 1. Characterization of tooth phenotype after Bmp2 deletion in a 3.6Col1a1Bmp2Cre-cKO (Bmp2-cKO^{od}) model. (Panel **A)** μCT analysis shows sample cross-sections of the represented colored parameters measured in teeth of 1-month- and 4-month-old control and Bmp2-cKO^{od} mice. For the first molars, colors are: pale blue, enamel; yellow, crown mineralized dentin; purple, pulp volume of crown; green, dentin volume of root; red, pulp volume in the root; and dark blue, periodontium. (Panel **B)** One-month-old Bmp2-cKO^{od} mice display significant ($P < 0.05$) decreases in crown dentin and root dentin volume ($n = 3$). (Panel **C)** X-rays of 1-month-old wild-type (Wt), heterozygote (Het), and Bmp2-cKO^{od} 1st molars show 50% thinner dentin (red arrow) in Bmp2-cKO^{od} mice compared with Wt and Het. Red asterisk, dental pulp; red arrow, mineralized dentin. The approximate 50% decrease in dentin thickness (by x-ray) is still observed in 8-month-old Bmp2-cKO^{od} mice compared with controls (data not shown).

Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH = 9.5). For alkaline phosphatase color development for 30 min at room temperature, we used 0.4 mL fresh, diluted NBT/BCIP Stock Solution (Roche) or 90 μL NBT Solution (Roche) and 70 μL BCIP (Roche) in 20 mL of buffer 3. With the procedure described above, most of our probes were of the same concentration (*i.e.,* Specific Activity—Dig molecules *per* ug of RNA) as the control probe after labeling (Appendix Fig. 5). The control probe was 0.1 μg/mL. Probes for future experiments were aliquoted at 1 μg (10 μL) in each tube and were stored at –80°C for up to 2 yrs. A fresh vial of probe for a given gene was used for each *in situ* hybridization experiment.

This procedure for determining the numerical relationship of blue to RNA concentration is as recommended by Roche. The far righthand dots, 1:100, 1:1000, and 1:10,000 dilutions (100-fold range), represent the approximate linear range for the signal and mRNA concentration. In some of our hybridizations, we were in the non-linear, saturated levels. This means that, in these cases, we underestimated the "true" change in mRNA expression level between, for example, control and Bmp2-cKO^{od}, especially genes such as Col1a1 and Dspp.

In situ Hybridization

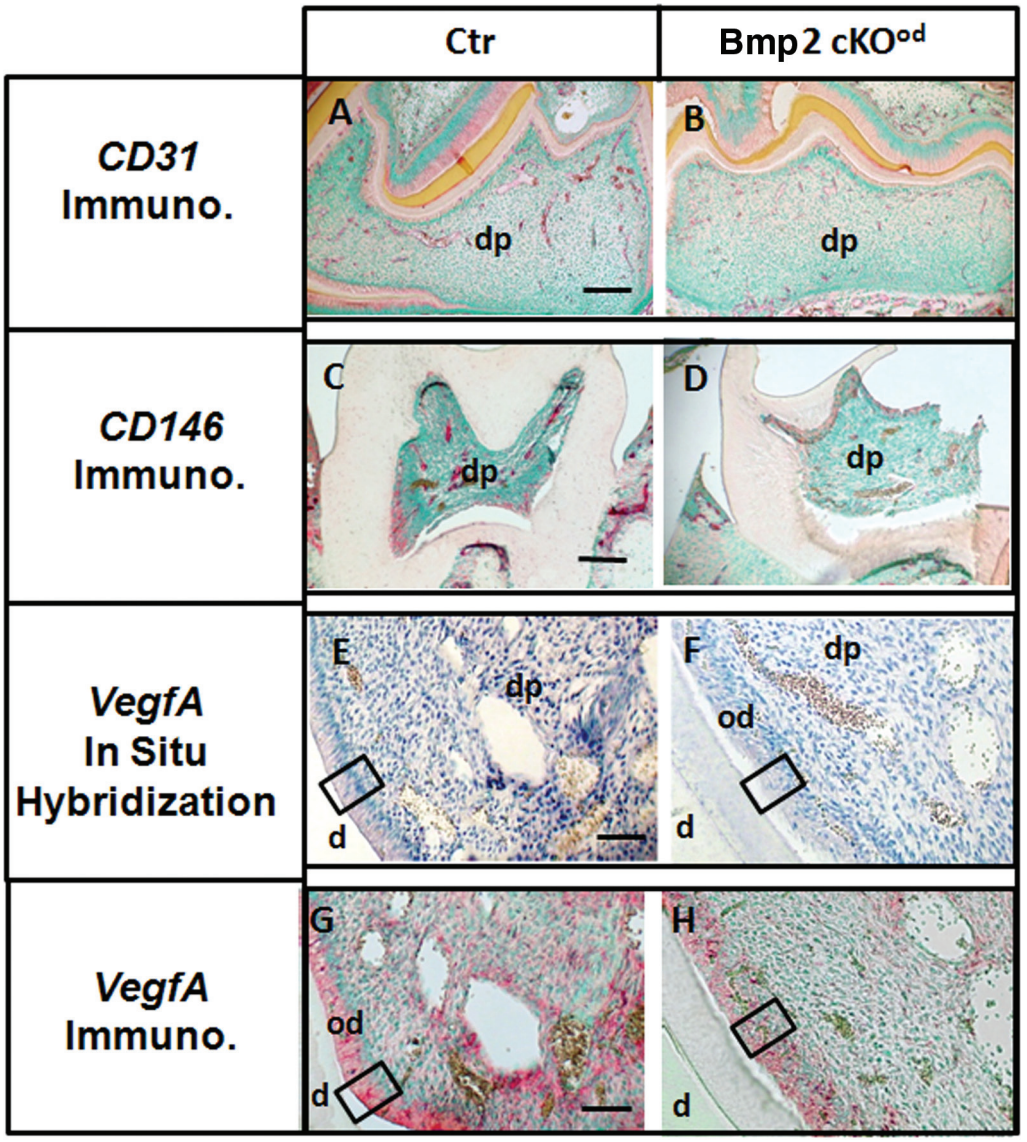
The *in situ* hybridization was performed with a modification of procedures described previously (Wilkinson, 1991, 1992, 1995; Sassoon and Rosenthal, 1993; Wilkinson and Nieto, 1993; Shibata *et al.*, 2000; Li *et al.*, 2005). Prior to hybridization, sections were deparaffinized with xylene and 100% ethanol following re-hydration. After treatment with proteinase K solution (5 mg/mL in 50 mM Tris, 5 mM EDTA, pH 7.6) for 10 min at 37°C, sections were re-fixed in 4% formaldehyde/PBS (0.2 M phosphate buffer, 3 M NaCl), acetylated (100 mM triethanolamine, 0.25% acetic anhydride), and pre-hybridized in 2 x SSC. Hybridization was performed at 55°C overnight, in the hybridization solution containing 50% formamide, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.3 M NaCl, 10% Dextran sulfate, 1X Denhardt's solution, 100 μg/mL denatured Salmon Sperm-DNA, 500 μ/mL tRNA), and 1 μg/mL UTP-digoxigenin-labeled RNA probe. After hybridization, the coverslips were removed in 5 x SSC at room temperature, and sections were washed in 50% formamide, 5 x SSC, and 1% SDS solution at 55°C 2 times for 30 min. The sections were incubated with RNase (40 mg/mL RNase A_1 and 10 U/mL RNase T_1) in RNase buffer solution (0.3 M NaCl, 10 mM Tris, 5 mM EDTA) at 37°C for 1 hr, followed by incubation in the same buffer without RNase for 30 min. Consecutive washes at 55°C were done twice with 50% formamide and 2 x SSC for 30 min, 3 times for 5 min each at room temperature in TBST (100 mM Tris, pH = 7.5, 150 mM NaCl, Tween-20 0.1%, Levamisole 2 mM), and once at room temperature in blocking mix (100 mM Maleic Acid, 150 mM NaCl, 10% Blocking reagent-Roche #1096176). Anti-digoxigenin antibody-alkaline phosphatase-labeled probe (Roche #1093274) was diluted 1:500 in blocking mix and was added after post-hybridization washing and blocking for overnight incubation at 4°C. The next day, the sections were washed in TBST for 5 min, in wash buffer (100 mM Maleic acid, 150 mM NaCl, Tween-20 0.1%, 2 mM Levamisole) twice for 15 min, and in pre-detection buffer (100 mM Tris, pH = 9, 100 mM NaCl, 2 mM Levamisole) for 5 min. Detection of hybridization signal was done by adding alkaline phosphatase substrate (NTB/BCIP, Roche #1681460) in detection buffer (10% polyvinyl alcohol 70-100 kDa, Sigma, 100 mM Tris, pH = 9, 100 mM NaCl, 2 mM Levamisole) overnight at 30°C. When detection of hybridization signal was completed, the sections were washed in water 3× for 5 min and counterstained with methyl green for 5 sec, dehydrated in ethanol, washed in xylene, and permounted.



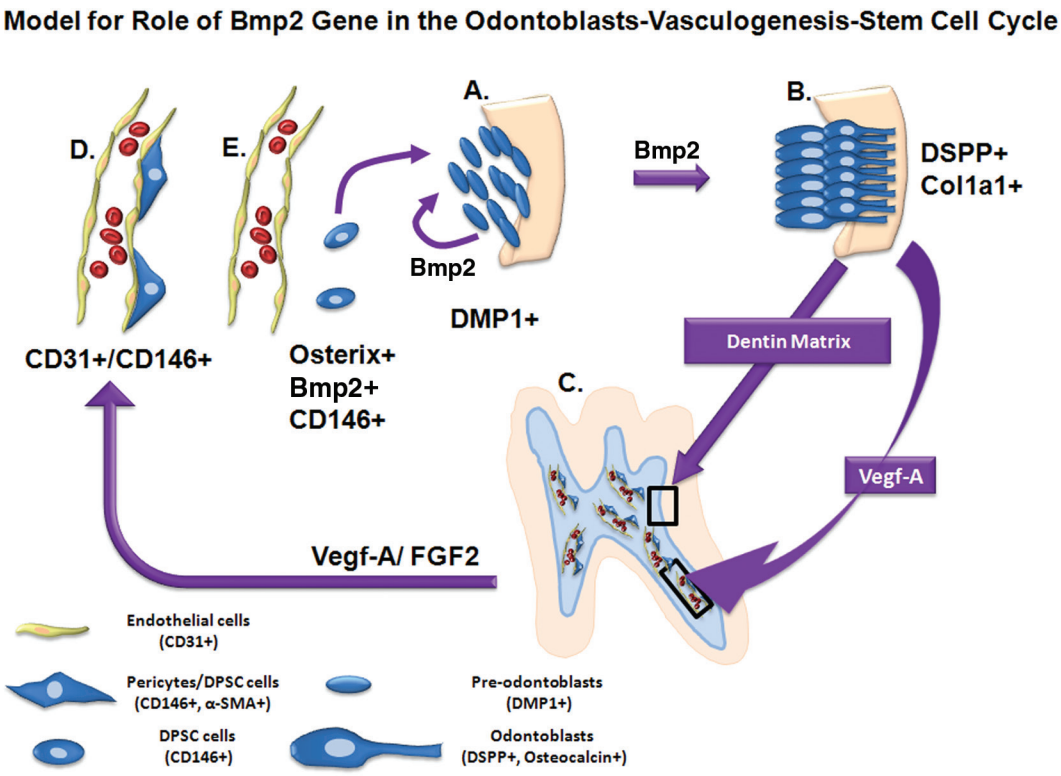
Appendix Figure 2. *Col1a1* and *Osterix* expression of the post-natal teeth in the Bmp2-cKO^{od} model. (Panels **A, B**) Col1a1 expression signal (blue) is reduced 75% in the incisor odontoblasts of the Bmp2-cKO^{od} compared with control 4-month-old animals, and expression level numerically estimated in **C**. Bar = 20 μm. (Panels **D, E**) *Osterix* expression signal (blue) shows a 60% reduced expression signal in molar odontoblasts, 5 days old, with reduced expression signal also noted in a subset of cells in the dental pulp and numerically estimated only in odontoblasts (Panel **F**). Bar = 20 μm. Dentin (d), odontoblasts (od), dental pulp (dp).

Numerical Estimation of Hybridization Signal with ImageJ

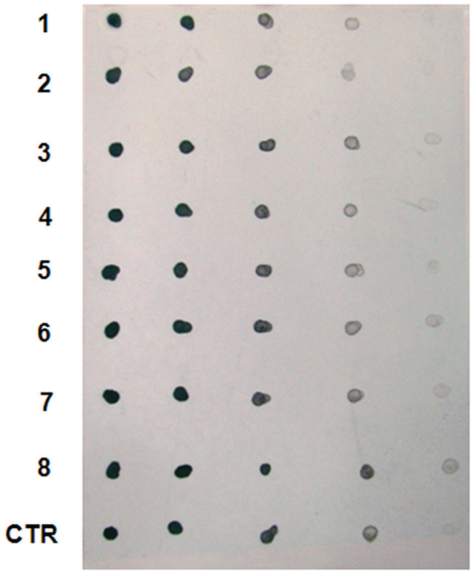
ImageJ is a public-domain, Java-based image processing program developed at the National Institutes of Health (NIH, Bethesda, MD, USA). ImageJ software is freely available for Microsoft Windows, Mac OS, Mac OS X, Linux, and Sharp Zaurus PDA and can be downloaded at http://rsb.info.nih.gov/ij/download.html. This software has been designed with an open architecture that provides extensibility *via* Java 1.1 version or later versions, with plugins and recordable macros. Custom acquisition, analysis, and processing plugins can be developed with ImageJ's built-in editor and a Java compiler. User-written plugins make it possible to solve many image processing and analysis problems, from 3-dimensional live-cell imaging to radiological image processing, and from multiple imaging system data comparisons to automated hematology systems. For estimating the *in situ* hybridization signal, we used purple-blue from NBT formazan, which is a product of alkaline phosphatase reaction from the use of BCIP and NTB substrate. The counterstain is a very light green (fast green), for minimum interference with the blue-purple mRNA expression signal. Before ImageJ is used, the area, minimum and maximum grey areas, and mean grey value can be selected in Set Measurements under Analyze. We used the tool "free hand selections" surrounding areas for measurements. All measurements were read, clicking on Measure under Analyze. All areas measured for a hybridization signal have to be carefully corrected for 2 types of background issues. First, several slides for every experiment processed through all the steps, but without a digoxigenin-labeled probe, must be included in background measurements. With these slides, fast green backgrounds and areas without any staining can be determined. Second, in the experiments with a digoxigenin probe, background measurements in areas of bone without any cells can be determined. These measurements should be compared with the 'no probe' levels. The appropriate 'no probe' signal in the equivalent cell area with the signal from the fast green counterstain must be determined and then subtracted from all the readings of the particular mRNA hybridization signal (or antiDIG antibody) to determine the "true" numerical estimated expression from a given probe.



Appendix Figure 3. *CD31, CD146, VegfA* mRNA, and *VegfA* protein are reduced in the Bmp2-cKO^{od} compared with controls. Endothelial cell marker CD31 (**A, B**) immunoreactivity in the Bmp2-cKO^{od} dental pulp is decreased compared with that in controls in 5-day-old animals. Bar = 100 μm. CD146 immunoreactivity (**C, D**) is reduced in the Bmp2-cKO^{od} mice in the dental pulp of the 1st molars of 1-month-old animals. Bar = 100 μm. *VegfA* overall mRNA expression signal (blue) in incisors of 4-month-old controls and Bmp2-cKO^{od} (**E, F**). Bar = 50 μm. The boxed areas in E and F are shown in Figs. 3E and 3F as higher magnification in the manuscript. (**G, H**) Overall immunoreactions of VegfA antibody in controls and Bmp2-cKO^{od}. Bar = 50 μm. The boxed areas in G and H are shown in Figs. 3G and 3H as higher magnifications in the manuscript. Dental pulp (dp), odontoblasts (od), and dentin (d).



Appendix Figure 4. Model for the role and mechanism of Bmp2 gene in post-natal tooth cytodifferentiation. The Bmp2 secreted by early pre-odontoblasts is required in an autocrine manner to drive proper odontoblast polarization **(A)**, dentinal tubule formation, and final terminal differentiation of the odontoblasts to produce high quality and quantity of dentin (**B, C**). In the process of terminal differentiation, the VegfA protein is secreted from mature odontoblasts into the pulp and, in combination with FGF2, stimulates the simultaneous production of endothelial cells and associated pericytes (**D**). The production of both pericyte and endothelial cells from a common mesodermal precursor, the mesenchymo-angioblast, is now supported by recent data (Vodyanik *et al.*, 2010). These pericytes on the blood vessels, in the pulp (and in the apical papilla and other unknown niches), and associated with vasculogenesis are the primary stem cells for crown and root odontoblasts, and VegfA and other signals activate the Bmp2 gene in these early dental pulp stem cells as they become Osterix+ and on their way to becoming pre-odontoblasts. This represents the odontoblast-vasculogenesis-stem cell cycle hypothesis in teeth and is worthy of further tests.



Appendix Figure 5. Numerical estimation of digoxigenin-labeled probes by dot blot and control labeled RNA from Roche. First row of dots (left) represents undiluted probes, followed by rows representing 1/10, 1/100, 1/1000, and 1/10,000 dilutions of probes. The concentration of the control probe is 0.1 μg/mL. In this example, our labeled probes (1-8) have concentrations similar to that of the control probe (CTR).

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