

Expression of ISG15 in Bone Marrow During Early Pregnancy in Ewes

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Abstract

Interferon-tau (IFNT) is the main signal for maternal recognition of pregnancy in ruminants. IFNT acts on the endometrium, corpus luteum, and liver through paracrine and endocrine style, which is involved in inhibiting the development of luteolytic mechanism and suppressing maternal immune rejection of the semi-allogeneic fetus. Bone marrow (BM) is a key component of the lymphatic system, supports the body's immune system through producing the lymphocytes. At present study, the BM was obtained from days 13, 16 and 25 of pregnant ewes, day 16 of non-pregnant ewes to study the expression of interferon stimulated gene 15 kDa protein (ISG15) mRNA and protein through a qRT-PCR assay, Western blot, and an immunohistochemistry analysis. Our results showed that the expression of ISG15 mRNA, proteins and conjugated proteins were up-regulated in the stroma of BM during early pregnancy, and the immunohistochemistry results confirmed that the ISG15 proteins were localized in the cytoplasm of different cells in the stroma of BM. In conclusion, IFNT derived from the conceptus induced up-regulated expression of ISG15 and conjugated proteins in the stroma of BM through an endocrine style, which were involved in regulating the maternal immune response during early pregnancy in ewes.

Keywords: Ewes, Bone marrow, Pregnancy, Interferon stimulated gene 15 kDa protein

Koyunların Erken Gebelik Döneminde Kemik İliğinde ISG15 Ekspresyonu

Özet

İnterferon-tau (IFNT) ruminantlarda gebeliğin maternal tanısındaki başlıca sinyaldir. IFNT endometrium, korpus luteum ve karaciğer üzerinde parakrin ve endokrin yollarla etki ederek luteolitik mekanizmanın gelişmesini inhibe eder ve semi-allojenik fetusun anne tarafından reddini baskılar. Kemik iliği lenfatik sistemin en önemli bileşenlerinden biri olup lenfositleri üretmek suretiyle vücudun bağışıklık sistemini desteklemektedir. Bu çalışmada, 13, 16 ve 25. günlerde gebe koyunlardan ve 16. günde gebe olmayan koyunlardan kemik iliği alınarak interferon tarafından uyarılan gen 15 kDa protein (ISG15) mRNA ve protein ekspresyonu qRT-PCR, Western blot ve immunohistokimyasal yöntemlerle araştırıldı. Elde edilen sonuçlar ISG15 mRNA, proteinler ile konjuge proteinlerin ekspresyonunun erken gebelik döneminde kemik iliğinde upregüle edildiğini ve immunohistokimyasal olarak ISG15 proteinlerin kemik iliğinin stromasında farklı hücrelerin sitoplazmasında yer aldığını göstermiştir. Sonuç olarak, gebeliğe bağlı olarak üretilen IFNT; ISG15 ve konjuge proteinlerin ekspresyonunu kemik iliğinin stromasında endokrin yolla upregüle etmiş ve böylece koyunların erken gebelik döneminde maternal bağışıklık cevabını düzenlenmesinde rol almıştır.

Anahtar sözcükler: Koyun, Kemik iliği, Gebelik; Interferon tarafından uyarılan gen 15 kDa protein

INTRODUCTION

It has been documented that interferon-tau (IFNT) is the main signal for maternal recognition of pregnancy in ruminants. IFNT acts on the endometrium to inhibit the development of luteolytic mechanism, and prolongs the lifespan of the ovarian corpus luteum (CL) to produce progesterone (P4) continuously ^[1]. It is reported that many interferon-stimulated genes are up-regulated in the ovine uterus during early pregnancy, such as 2',5'-oligoadenylate

synthetase (OAS-1) ^[2], ubiquitin-like interferon-stimulated gene 15-kDa protein (ISG15) ^[3], Mx proteins ^[4], Stat-1, Stat-2, interferon regulatory factor 1 (IRF-1) and IRF-9 ^[5]. In addition, interferon-stimulated genes are also up-regulated in the ovine CL and blood cells during early pregnancy, including ISG15, OAS-1 ^[6], receptor transporter protein 4 (RTP4) ^[7]. It is through an endocrine style that IFNT secreted by the conceptus releases into the uterine vein, and extends luteal life span in ewes ^[8]. It is also reported that ISG15 and MX-1 mRNAs are detectable in the liver on day 18 of pregnancy in cattle, which is induced by IFNT ^[9].



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As an ubiquitin cross-reactive protein, ISG15 conjugates to the target proteins by its C-terminal LRLRGG motif. ISG15 modification (ISGylation) does not target proteins for degradation, but enhances the cellular response to interferon through enhancing Jak-1 and Stat-1 activities^[10], which is different from ubiquitination. ISG15 plays a role in innate immunity, and is closely regulated by specific signaling pathways. During ruminant pregnancy, the maternal immune system must inhibit its immune rejection of the semi-allogeneic fetus to adapt the existence of the conceptus^[11,12]. Bone marrow (BM) is a key component of the lymphatic system, supports the body's immune system through producing the lymphocytes. It is reported that there is a progressively diminished in the numbers of pre/pro B and immature B cells in the BM of pregnant mice^[13]. However, it is unclear that the expression of ISG15 in the BM during early pregnancy in sheep. In this study, the BM from non-pregnant and early pregnant ewes were sampled to explore the expression of ISG15, which may be helpful to make out the immune suppression mechanism regulated by BM during early pregnancy in sheep.

MATERIAL and METHODS

Animals and Experimental Design

Small Tail Han ewes approximately 18 months of age with normal oestrous cycles were observed daily for oestrus using vasectomized rams, and were mated three times with intact rams at 12-h intervals after the detection of sexual receptivity. The experimental protocol was approved by the Hebei University of Engineering Animal Ethical Committee (HUEAE 2015-021), and humane animal care and handling procedures were followed throughout the experiment. The day of coition was counted as day 0 of pregnancy, and the ewes were randomly divided into four groups ($n = 5$ for each group), and ewes assigned to the non-pregnant group were not mated with intact ram. The BM was obtained from ewes on days 13, 16 and 25 of pregnancy, day 16 of non-pregnancy at the time of slaughter. Pregnancy was confirmed through observing the presence of conceptus in the uterus. The BM was sampled from the femur, and several sections of BMs (0.3 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in PBS buffer (pH 7.4). The remaining portions of BMs were frozen in liquid nitrogen for subsequent quantitative Real Time PCR (qRT-PCR) and protein analysis.

RNA Extraction and qRT-PCR Assay

The total RNA from the BM sample was extracted using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen, California, USA), and the cDNA was synthesized with FastQuant RT Kit (Tiangen Biotech Co., Ltd., Beijing). The SuperReal PreMix Plus Kit (Tiangen Biotech Co., Ltd., Beijing) was employed for qRT-PCR. The primer sequences of ISG15 (accession no. NM_001009735.1) was

Gene	Primer	Sequence	Size (bp)
ISG15	Forward	CATCCTGGTGAGGAACGACAA	186
	Reverse	AAAGACAGCCAGAACTGGTCC	
GAPDH	Forward	GGGTCATCATCTCTGCACCT	176
	Reverse	GGTCATAAGTCCCTCCACGA	

designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1), and GAPDH was based on Oliveira et al.^[6] (Table 1). The relative levels of mRNA expression were calculated using an internal control gene (GAPDH). The relative expression value was set as 1 for the group of day 16 non-pregnant BMs.

Western Blot

The total proteins of the BMs were extracted by RIPA Lysis Buffer (Biosharp, BL504A). The protein concentration was measured using a BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, PA115) with bovine serum albumin as the standard. An equal amount of total proteins ($10 \mu\text{g}/\text{lane}$) was separated using 12% SDS-PAGE, and the proteins were transferred to $0.22 \mu\text{m}$ polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The PVDF membranes were blocked in 5% non-fat milk in Tris-buffered saline plus Tween 20 (TBST) at 4°C overnight. The mouse anti-ovine ISG15 monoclonal antibody was prepared as described by Austin et al.^[14] with roISG15 instead of rboISG15. ISG15 and conjugated proteins were detected using the ISG15 monoclonal antibody (1:20000) at 37°C for 1 h. The secondary goat anti-mouse IgG-HRP was at a dilution 1:20000 (37°C for 1h). Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd, Beijing) was used to detect immunoreactive bands. Sample loading was monitored with the GAPDH antibody (Santa Cruz Biotechnology, Inc., sc-20357) at a dilution of 1:1000. Immunoreactive proteins greater than 30 kDa were deemed conjugates^[15] and semi-quantified together. The intensity (INT) of blots were quantified using Quantity One V452 (Bio-Rad Laboratories), and the unit was INT/mm^2 .

Immunohistochemistry Analysis

The fixed BMs were embedded in paraffin, and paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. The sections were quenched endogenous peroxidase activity with 3% H_2O_2 , and reduced non-specific binding was completed with 5% normal goat serum in PBS buffer. Immunocytochemical localizations of ISG15 in the BMs were performed using the ISG15 monoclonal antibody (1:2000). The immunoreactive protein was detected using secondary goat anti-mouse IgG-HRP (1:1000). A DAB kit (Tiangen Biotech Co., Ltd., Beijing) was used to visualize the antibody binding sites in the tissue sections, and the nucleus was stained with hematoxylin. Finally, the images were captured on using a light micro-

scope (Nikon Eclipse E800, Japan) and a digital camera DP12, and the intensity of staining and density of stained cells were analyzed through the images.

Statistical Analyses

The relative expression values for the qRT-PCR assay were calculated using the $2^{-\Delta\Delta Ct}$ analysis method. Statistical analyses were performed using Statistical Analysis System Package version 9.1 for Windows (SAS Institute, Cary, NC, USA). P value < 0.05 was considered statistically significant difference.

RESULTS

Relative Expression Level of ISG15 mRNA in BMs

The expression levels of ISG15 mRNA were significantly higher at days 13, 16 and 25 in pregnant BMs comparing with that at day 16 in non-pregnant BMs ($P < 0.05$), and there was a statistical difference between days 13 and 25 of pregnant BMs in the expression levels of ISG15 mRNA. Furthermore, the relative expression level of ISG15 mRNA was significantly higher at day 16 in pregnant BMs than that at days 13 and 25 in pregnant BMs in sheep (Fig. 1).

Western Blot Analysis for the ISG15 Proteins in BMs

Western blot analysis showed that the ISG15 and conjugated proteins were expressed at days 13, 16 and 25 in pregnant BMs, and only the ISG15 conjugated proteins were expressed at day 16 in non-pregnant BMs. Western blot analysis revealed that the ISG15 and conjugated

proteins up-regulated increasingly from day 13 to day 16 in pregnant BMs, and down-regulated from day 16 to day 25 in pregnant BMs in ewes (Fig. 2).

The Immunohistochemistry Analysis for ISG15 in BMs

The immunohistochemistry does not distinguish between free ISG15 and conjugated proteins in the tissue sections, because the antibody recognizes the both forms of free ISG15 and conjugated proteins. In this study, pregnant ewes were adult, so the BMs from femurs were converted to yellow marrow. Yellow marrow is mainly made up of fat cells and little stroma, and stroma includes fibroblasts, macrophages, osteoblasts, osteoclasts, and endothelial cells. Immunohistochemistry analysis confirm that the ISG15 and conjugated proteins were expressed in the stroma from days 13, 16 and 25 in pregnant BMs, and day 16 in non-pregnant BMs. The staining intensity was the strongest in the stroma from day 16 of pregnant BMs, and the staining intensity of the stroma from day 16 in non-pregnant BMs was the lowest (Fig. 3). Furthermore, there was a strong staining intensity located in the cytoplasm of different cells in the stroma from pregnant BMs, including macrophages.

DISCUSSION

It is necessary for the maternal immune system to suppress maternal immune rejection of the semi-allogeneic fetus during pregnancy, which is essential for the conceptus to develop normally in ruminants [11,12]. Many researchers reported that the IFNT exists its effect on blood cells [7,16-18], CL [6,19-21], and liver [9] in the ovine and bovine. It is through paracrine and endocrine style that IFNT secreted by the conceptus releases into the uterine vein, and extends the luteal life span during early pregnancy in sheep [8,22,23]. Therefore, it is possible that IFNT exerts its effect on the BM, which is implicated in regulating the maternal immune system through blood circulation during early pregnancy in sheep.

Our results revealed that there were changed expression of ISG15 mRNA, ISG15 and conjugated proteins from day 13 to 25 in pregnant BMs (Fig. 1 and Fig. 2). These findings were similar to previous results that there were changed expressions of ISG15 in endometrium [3,24], blood cells [25,26], CL [6,27] during early pregnancy in sheep and cattle. It was indicated that the embryonic signal (IFNT) reached the blood cells, CL and BM to up-regulate ISG15 expression through blood or/and immune cells

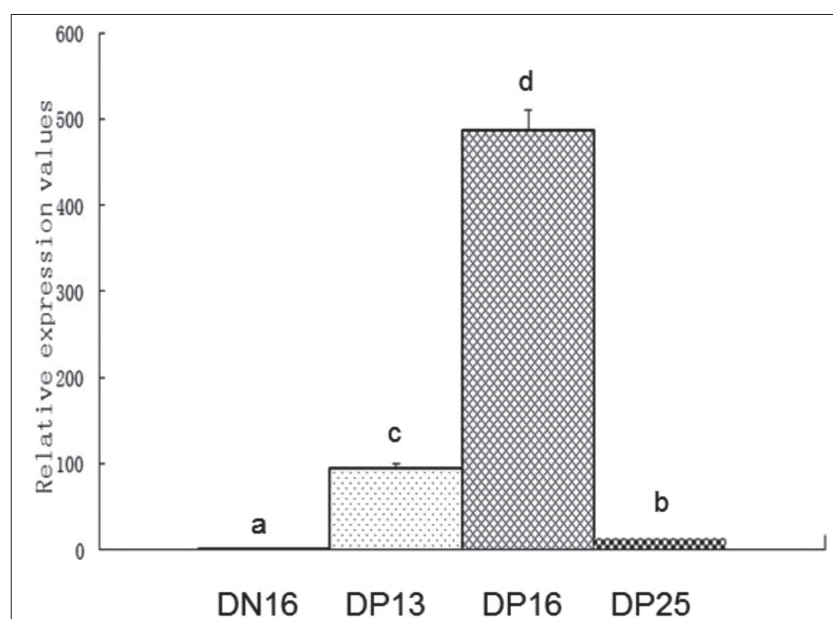


Fig 1. Relative expression value of ISG15 mRNA in day 16 non-pregnant, days 13, 16 and 25 pregnant bone marrow through quantitative real time PCR in sheep. Note: DN16 = Day 16 of non-pregnant bone marrow (BM); DP13 = Day 13 of pregnant BM; DP16 = Day 16 of pregnant BM; DP25 = Day 25 of pregnant BM. Significant difference was indicated by different letters within different column

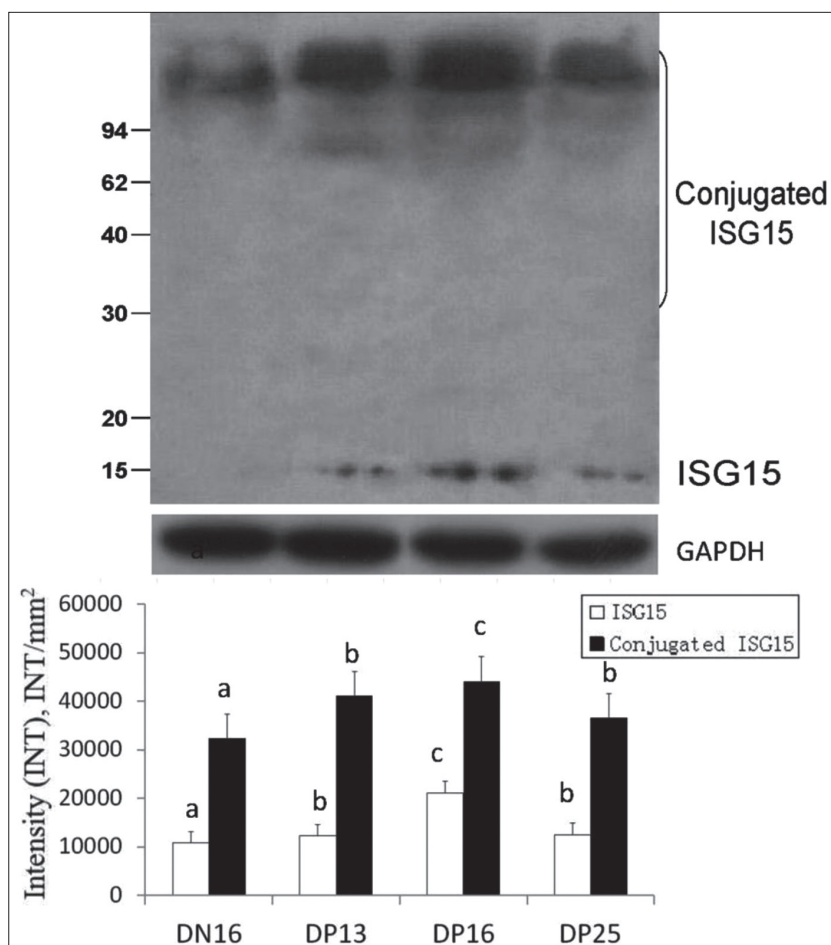


Fig 2. Expression of ISG15 and conjugated proteins in ovine bone marrow from day 16 of non-pregnancy, days 13, 16 and 25 of pregnancy. Note: DN16 = Bone marrow (BM) from day 16 of the estrous cycle; DP13 = BM from day 13 of pregnancy; DP16 = BM from day 16 of pregnancy; DP25 = BM from day 25 of pregnancy. Immunoreactive proteins greater than 30 kDa were deemed ISG15 conjugated proteins. Significant differences ($P < 0.05$) are indicated by different superscript letters within same color column

during early pregnancy in sheep. However, there was no expression ISG15 protein in mammary gland during early pregnancy in cattle [27], and there was also no significant change in expression of ISG15 mRNA in cells from milk samples during early pregnancy in cows [28]. Furthermore, we also found that there was no expression ISG15 protein in lymphoid node and spleen during early pregnancy in ewes (our unpublished data), so IFNT exerted its effect selectively on different tissues and cells during early pregnancy in sheep. Therefore, we had no clear explanation that IFNT in the circulation selectively influenced expression of ISG15 by different immune organs during early pregnancy in sheep. It was probable that BM was in some part responsive to the circulating IFNT, or/and peripheral blood immune cells, which remained to be elucidated and verified.

As the central immune organ, BM produces lymphocytes to support the body's immune system. The immune cells mediate a cross-talk between the embryo and mother during early pregnancy in human [29]. There were

significantly higher rate of preterm delivery and lower birthweight babies in female who conceived naturally after peripheral blood or BM transplantation than normal conception [30], which indicated that BM was of importance for normal conception. As an ubiquitin cross-reactive protein, ISG15 contains two domains with structural homology similar to ubiquitin [31]. Unlike ubiquitination, protein ISG15 modification (ISGylation) does not target proteins for degradation, but regulates the function of target proteins [32], and ISGylation plays a role in innate immunity [33]. Our results showed that there was an up-regulation of ISG15 and conjugated proteins in the BMs (Fig. 2), which indicated that BM was implicated in regulating the maternal immune response during early pregnancy in ewes.

We also reported that there were changing expressions of Th1 and Th2 cytokines in bovine peripheral blood mononuclear cells (PBMC) during early pregnancy [34]. It is known that macrophages play a crucial role in initiating the immune response. Our present study showed that the positive signal for ISG15 was strongly localized to the cytoplasm of macrophages (Fig. 3). It was suggested that IFNT derived from the conceptus entered the maternal blood circulation, which induced up-regulated expression of ISG15 and conjugated proteins in the stroma of BM. The immune

cells from the stroma of BM up-regulated expression of ISG15 and conjugated proteins, and then the immune cells entered into the maternal blood circulation, which was implicated in regulating the maternal immune response. Therefore, it was suggested that up-regulated expression of ISG15 and conjugated proteins in the stroma of BM may be of importance in pregnancy maintenance in ewes.

In conclusion, the expression of ISG15 mRNA, proteins and conjugated proteins were up-regulated in the ovine BM during early pregnancy, which was suggested that IFNT derived from conceptus entered into the maternal blood circulation, induced up-regulated the expression of ISG15 and conjugated proteins in the stroma of BM. The immune cells from the stroma of BM were up-regulated expression of ISG15 and conjugated proteins, which were involved in regulating the maternal immune response through an endocrine style during early pregnancy in ewes.

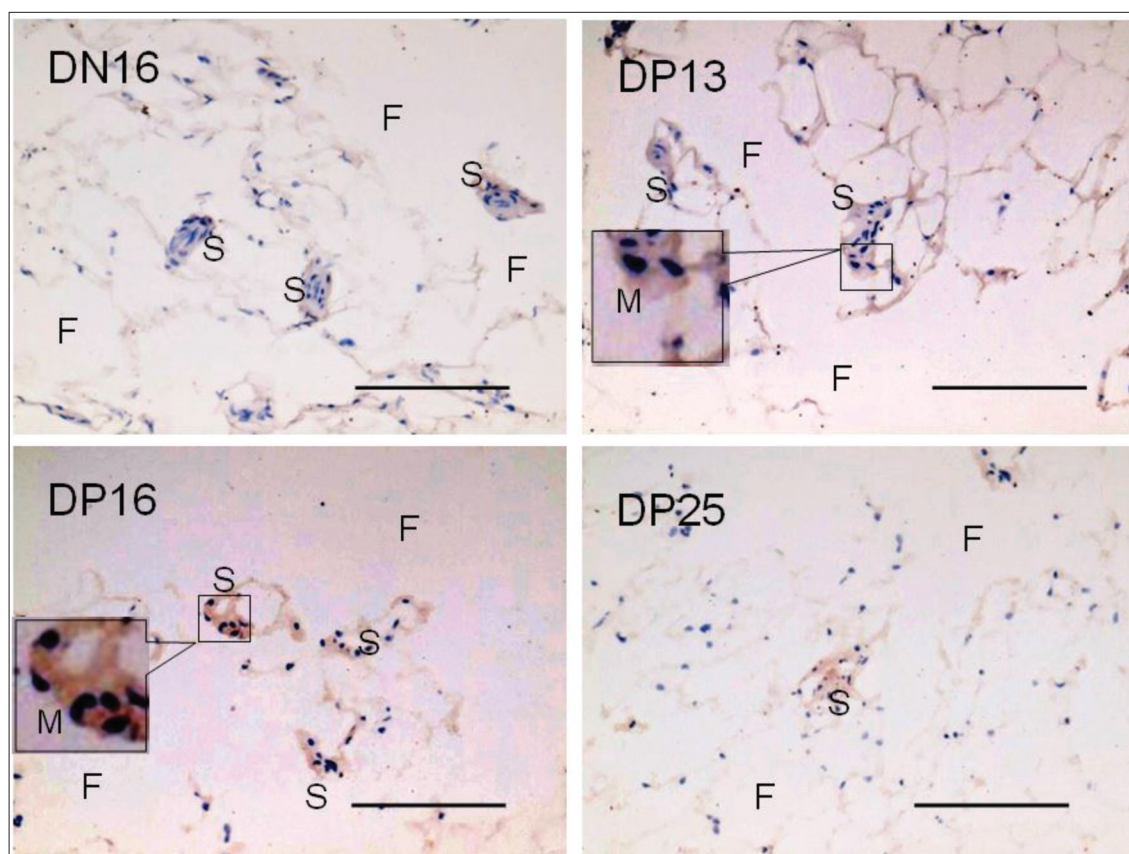


Fig 3. Representative immunohistochemical localization (200 × magnification) of ISG15 in ovine bone marrow collected on day 16 of the estrous cycle and days 13, 16 and 25 of pregnancy. The macrophages are conspicuous by their size and irregular nucleus. The immunohistochemistry does not distinguish between free ISG15 and conjugated proteins in the tissue sections. Note: DN16 = Bone marrow (BM) from day 16 of the estrous cycle; DP13 = BM from day 13 of pregnancy; DP16 = BM from day 16 of pregnancy; DP25 = BM from day 25 of pregnancy; S = Stroma; F = Fat; M = macrophages. Bar = 50 μm

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