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Haploinsufficiency of E-selectin ligand-1 is Associated with Reduced Atherosclerotic Plaque Macrophage Content while Complete Deficiency Leads to Early Embryonic Lethality in Mice

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Abstract

E-selectin-1 (ESL-1), also known as golgi complex-localized glycoprotein-1 (GLG1), homocysteine-rich fibroblast growth factor receptor (CGR-1), and latent transforming growth factor-β complex protein 1 (LTCP-1), is a multifunctional protein with widespread tissue distribution. To determine the functional consequences of ESL-1 deficiency, mice were generated carrying an ESL-1 gene trap. After backcrossing to C57BL6/J for 6 generations, mice heterozygous for the gene trap (*ESL-1*$^{+/-}$) were intercrossed to produce *ESL-1*$^{-/-}$ mice, however *ESL-1*$^{-/-}$ mice were not viable, even at embryonic day E10.5. To determine the effect of heterozygous ESL-1 deficiency on atherosclerosis, apolipoprotein E deficient (*ApoE*$^{-/-}$), *ESL-1*$^{+/-}$ mice were generated and fed western diet. Compared to *ApoE*$^{-/-}$, *ESL-1*$^{++}$ mice, atherosclerotic lesions from *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ contained more collagen and fewer macrophages, suggesting increased plaque stability. In conclusion, heterozygous deficiency of ESL-1 is associated with features of increased atherosclerotic plaque stability while complete deficiency of ESL-1 leads to embryonic lethality.

Keywords

leukocyte; selectins; macrophage; atherosclerosis; endothelium

Introduction

E-selectin ligand-1 (ESL-1) is a leukocyte ligand that binds to the endothelial selectin, E-selectin, to mediate leukocyte-endothelial interactions[1]. ESL-1 has also been named golgi complex-localized glycoprotein-1 (GLG1)[2], cysteine-rich fibroblast growth factor receptor (CGR-1) [3], and latent transforming growth factor-β complex protein 1 (LTCP-1)[4] because of its many additional protein interactions and widespread tissue distribution.

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Authorship Contributions
Contribution: D.T.E. designed research and wrote the paper; W.L. designed research, performed experiments, analyzed data, and wrote the paper; H.W, C.G., J.W., J. K., and K.L.B. performed experiments.

Disclosures
None.

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There have been at least 2 recent previous reports of mice with genetic deficiency of ESL-1[5, 6]. In one report, mice with genetic deficiency of ESL-1 were generated using a targeting vector designed to delete exons 13-16 of ESL-1. Mice homozygous for this mutation were 30-50% smaller than wild type (WT) littermates and this phenotype was associated with increased TGF-β signaling in the growth plates of bones leading to reduced chondrocyte proliferation and delayed terminal differentiation[5]. In another study using a gene trap approach, mice homozygous for the gene trap were present at the expected mendelian ratio at embryonic day 18.5 but there was 90% loss by postnatal day 2[6].

To further investigate the consequences of ESL-1 deficiency, we generated a mouse model of ESL-1 deficiency using a gene trap approach and assessed viability of ESL-1 null mice and vascular consequences of ESL-1 haploinsufficiency.

Methods

Mice

Apolipoprotein E deficient (*ApoE*$^{-/-}$) and C57BL6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice carrying an ESL-1 gene trap were generated after injection of targeted embryonic stem (ES) cells (International Gene Trap Consortium, Sequence Tag RST092) into C57BL6/J recipients. Rapid amplification of cDNA ends (RACE) kit (Invitrogen, Grand Island, NY) was used to identify adjacent exon. PCR primers were then designed to amplify the mutant allele with one primer residing within the gene trap (Primer 3 5'-GAGACCCTGGCCTCCTGATTC-3') and the other located in the adjacent endogenous *ESL-1* (exon 1) sequence (Primer 1 5'-CCCCAAACACACCTGGAGCAACAA-3'). The wild-type *ESL-1* sequence was amplified between exon 1 (primer 1 5'-CCCCAAACACACCTGGAGCAACAA-3') and intron1 (Primer 2 5'-CACAAAAGACAGGAGCGCAAACAG-3'). Mice carrying the gene trap were serially backcrossed for 6 generations to the C57BL6/J strain and then intercrossed to produce offspring homozygous for the gene trap. Timed matings were used for analyses of embryos. For atherosclerosis study, both *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice were fed on Western diet (Rodent Western Diet #D12079B, Research Diet, New Brunswick, NJ) for 9 weeks beginning at 4 weeks of age. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the University of Michigan Committee on Use and Care of Animals.

RNA analysis

RNA preparation from spleen for ESL-1 expression was analyzed as previously described [7]. Liver was source of RNA for analysis of CD68. ESL-1 and CD 68 RT PCR primers were purchased from Applied BioSystems (Foster City, CA) and the 2-ΔΔCT method [8] was used to analyze results.

Immunohistochemistry

Liver tissue was collected and fixed in zinc formalin. Macrophages in paraffin embedded liver sections were identified with a rat anti-mouse F4/80 monoclonal antibody (1:400) (Abcam, Cambridge, MA) followed by detection with biotin-conjugated secondary goat anti-rat IgG (1:100) (Accurate Chemical & Scientific Corp., Westbury, NY).

ESL-1 Western blotting

Transblotted PDVF membranes were incubated with ESL-1 antibody (1:10,000, 5% milk in PBST, rabbit IgG), then IRDye 800CW Donkey anti-Rabbit IgG (1:10,000, LI-COR Biotechnology, Lincoln, Nebraska). Protein was detected using an Odyssey Imaging System

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(LI-COR Biotechnology, Lincoln, Nebraska). Actin was used as a housekeeping protein to normalize the data.

Atherosclerosis model

For atherosclerosis studies, *ApoE*$^{-/-}$,*ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$,*ESL-1*$^{+/+}$ mice were generated by serial intercrosses between *ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$ mice. Quantification of atherosclerosis was performed as previously reported[9]. Lesion macrophages and smooth muscle cells were quantified by immunohistochemistry with rat anti-mouse CD68 (FA-11) antibody (1:200 dilution; AbD Serotec, Raleigh, NC) and α-smooth muscle cell actin antibody (1:1000 dilution, Chemicon), followed by detection with biotin-goat anti-rat IgG (Accurate Chemical & Scientific Corp., Westbury, NY). The area of lesion occupied by collagen was determined after staining with Sirius red (Sigma, St. Louis, MO).

Thioglycollate-induced peritonitis

Leukocytes were harvested from the peritoneum ESL-1+/- and ESL-1+/+ mice 4 days after IP injection of 2 mL (4% wt/vol) thioglycollate broth. The collected peritoneal cells were incubated with ACK lysing buffer (Lonza, Hopkinton, MA) to remove red blood cells. Total numbers of leukocytes were counted manually by hemocytometer (Sigma Chemical, St. Louis, MO) according to manufacturer's instruction.

Intravital microscopy

Intravital microscopy was performed as previously published[7]. Three venules were analyzed from each mouse.

Pressure myography

Endothelial function was measured via pressure myography on 18 week old mice which were fed 10 weeks of high-fat, high-sucrose diet (D12451, Research Diet Inc, New Brunswick, NJ). Mesenteric arteries were removed and vascular activity was measured as previously described[10]. Endothelial-dependent relaxation was assessed by measuring the dilatory response to acetylcholine (Ach) (Sigma–Aldrich, St. Louis, MO) (10^{-9} to 10^{-4} mol/L) in norepinephrine (NE) (Sigma–Aldrich, St. Louis, MO) pre-contracted vessels (10^{-5} mol/L) at 45mm Hg intraluminal pressure.

Statistical analyses

Chi square was used to determine significance of ESL-1 genotype proportions. Results from other experiments are expressed as mean ± SEM. The statistical significance of differences between groups was determined by Student's *t* test. Values of $p<0.05$ were considered significant.

Results

Characterization of ESL-1 deficient mice

Following RACE, amplified PCR fragments were sequenced and aligned with more than 300 base pairs of ESL-1 exon 1 sequence (Figure 1A).

No ESL-1 null mice (*ESL-1*$^{-/-}$) were present at 3 weeks of age while expected numbers of *ESL-1*$^{+/-}$ and *ESL-1*$^{+/+}$ mice were present (Figure 1B), consistent with complete lethality due to ESL-1 deficiency. Timed breedings between *ESL-1*$^{+/-}$ mice were set up to determine at what stage lethality occurred. *ESL-1*$^{+/-}$ females were mated to *ESL-1*$^{+/-}$ males and pregnant females were then sacrificed at gestation day 10.5. DNA was analyzed from embryos with primers to amplify the gene trap and wild-type alleles. No *ESL-1*$^{-/-}$ embryos

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were identified (Figure 1B). These findings are consistent with a critical role of ESL-1 in early embryonic development.

To determine the effect of heterozyogous ESL-1 deficiency on ESL-1 expression, ESL-1 RNA levels were measured by quantitative RT-PCR from spleen RNA. RNA expression was significantly lower in *ESL-1*$^{+/-}$ mice compared to *ESL-1*$^{+/+}$ mice (1.10 ± 0.09 versus 6.03 ± 2.72 relative expression, n=3 mice per group, p=0.03). ESL-1 protein levels, detected via Western blotting, were also lower in *ESL-1*$^{+/-}$ mice compared to *ESL-1*$^{+/+}$ mice (Figure 1C).

To determine the effect of heterozygous ESL-1 deficiency on leukocyte-endothelial (L-E) interactions, intravital microscopy was performed on the cremaster venules of 8 week old male *ESL-1*$^{+/-}$ mice following challenge with locally injected tumor necrosis factor-alpha (TNF-α). *ESL-1*$^{+/-}$ mice demonstrated increased numbers of rolling leukocytes but decreased firmly attached leukocytes on endothelial cells compared to *ESL-1*$^{+/+}$ littermates (Figure 1D).

To determine the effect of heterozygous ESL-1 deficiency on endothelial function, pressure myography[10] was performed on mesenteric arteries from 18 week old mice which were fed 10 weeks of high-fat, high-sucrose diet. Endothelial dependent relaxation response to acetylcholine (10^{-4} mol/L) was impaired to the same extent in both *ESL-1*$^{+/-}$ and *ESL-1*$^{+/+}$ littermate mice (29.42±3.57 versus 24.46±6.21 %, respectively).

To assess the role of heterozygous ESL-1 deficiency on leukocyte extravasation, 10 weeks old *ESL-1*$^{+/-}$ and *ESL-1*$^{+/+}$ mice were challenged with peritoneal thioglycollate injection. Compared to *ESL-1*$^{+/+}$ mice, intraperitoneal leukocytes were reduced in *ESL-1*$^{+/-}$ mice ($6.36±1.21×10^6$ versus $2.71±0.61×10^6$ cells/animal, n=4 mice per group, p=0.04).

Effect of reduced ESL-1 expression on atherosclerosis

To determine if heterozygous deficiency of ESL-1 would affect monocyte recruitment to atherosclerotic plaques, *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice were compared. Following 9 weeks of a western diet, atherosclerosis was quantified by oil-red-o enface staining of the aorta and major branches. No difference in surface area staining was noted between the groups of mice (*ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ = 2.22±0.25 (n=8) versus *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ = 2.37±0.37% (n=14) lesion area, p=0.1). Similarly, lesion thickness quantified at the level of the aortic valve was not different between *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice (2245.81±380.93 versus 1663.88±374.45 μm^2, respectively, p=0.72). However, lesion analysis revealed greater collagen deposition (Figure 2A-2C) and fewer macrophages (Figure 2D-2F) in *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ mice compared to *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice. Smooth muscle cell alpha actin staining of the aortic root was not different between *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice (2.10 ± 0.56 versus 3.80 ± 1.66 %, respectively, p=0.1).

Reduced CD68 expression was also observed in liver tissue of *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ compared to *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice (1.24±0.11 versus 1.69±0.11 respectively, n=4-5 per group, p=0.026), and this correlated with reduced macrophage content (7.55±0.96 cells per 400x field in *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ compared to 13.58±1.37 cells per 400x field in *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice, n=6-7 per group, p< 0.0005).

No significant differences in LV chamber thickness were observed between *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ mice (577.7±31.73 versus 582.1±26.85 μm, respectively, n=5 and 8 per group) or right ventricular chamber thickness (274.0±7.31 versus

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313.7±24.1μm, respectively, n=5 and 8 per group). Myocardial collagen deposition was also not different between *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ and *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ by Sirius Red staining.

Discussion

ESL-1 is a multifunctional protein shown to be involved in the regulation of leukocyte-endothelial interactions[11]. ESL-1 also has been shown to regulate TGF-beta activity and FGF signaling[6]. Its localization in the golgi suggest many other cellular functions may also be affected by ESL-1 [12]. Accordingly, genetic ESL-1 deficiency states lead to severe phenotypes ranging from growth retardation [5] to perinatal death[6]. In the current study, the effects of ESL-1 deficiency are confirmed to be severe as the construct used in this study led to early embryonic lethality in mice homozygous for the gene trap. Heterozygous mice developed normally with no apparent phenotypic changes in the unchallenged state.

Knockdown of ESL-1 expression using shRNA sequences in leukocytes transfected with lentivirus has previously been shown to result in a partial reduction in leukocyte binding to E-selectin [11]. Other relevant leukocyte ligands to E-selectin include CD44 and Psgl-1[13, 14]. Analysis of cremaster venules revealed that ESL-1 knockdown either did not affect or increased leukocyte rolling frequencies, in the presence or absence of CD44, respectively, while the adherent leukocyte fraction was decreased by ESL-1 deficiency [11]. Deficiency of E-selectin has also been associated with increased rolling leukocyte flux fraction and reduced firm adherence [15]. In the current analysis of L-E interactions, ESL-1 haplo-insufficiency was associated with increased leukocyte rolling and reduced firm adherence, supporting the role of ESL-1 in promoting the transition from leukocyte rolling to firm attachment on endothelial cells. This L-E interaction pattern was associated with reduced leukocyte extravasation following stimulation with intraperitoneal thioglycollate.

In the chronic, low grade inflammatory condition of atherosclerosis, L-E interactions have been associated with increased monocyte influx into the wall of large vessels, leading to acceleration of atherosclerosis[16]. Although ESL-1 haploinsufficiency did not affect total atherosclerotic burden, it did affect lesion composition, with reduced macrophage content and increased collagen content, which may have important implications regarding stability of the plaque. Increased collagen deposition could be due to dysregulation of TGF-B in *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ mice[17]. Whether complete ESL-1 deficiency would have a more significant effect on atherosclerotic plaque progression will require additional studies with conditional knock-out mice. In addition, we cannot rule out the possibility that differences in atherosclerosis might be observed at different ages and/or with different dietary challenges. Consistent with reduced monocyte trafficking, reduced hepatic macrophage content due to ESL-1 haploinsufficiency was also noted in *ApoE*$^{-/-}$, *ESL-1*$^{+/-}$ compared to *ApoE*$^{-/-}$, *ESL-1*$^{+/+}$ mice.

Although no differences in collagen staining in the myocardium of these atherosclerotic-prone mice were observed, we cannot exclude an important effect of ESL-1 on myocardial remodeling following induction of MI or hypertrophy.

In conclusion, ESL-1 deficiency is associated with early embryonic lethality; while haploinsufficiency is sufficient to affect L-E interactions and reduce leukocyte trafficking in both acute and chronic models of inflammation. Additional studies with conditionally mutant mice will be needed to determine whether ESL-1 is a safe and effective target for modifying inflammatory diseases.

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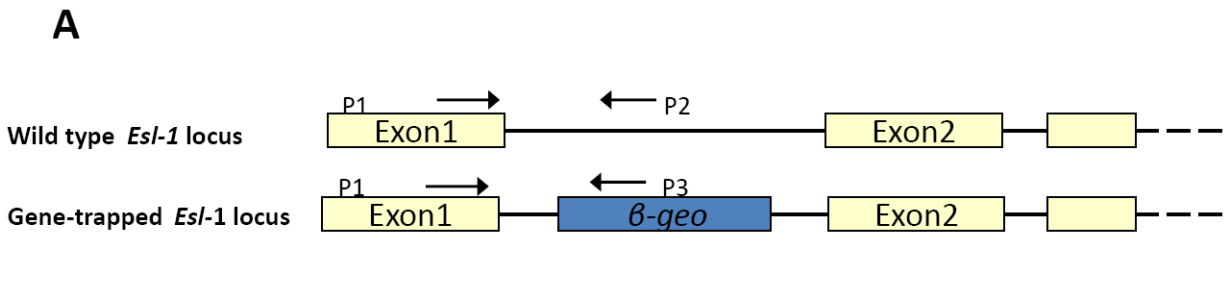
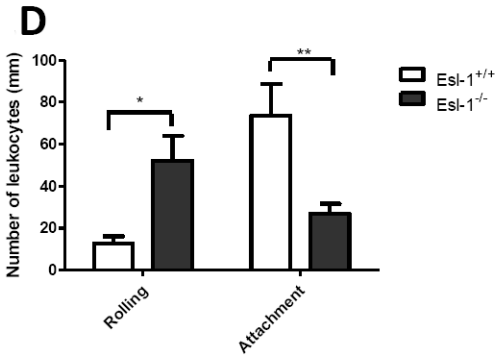


B

	Esl-1$^{+/+}$	Esl-1$^{+/-}$	Esl-1$^{-/-}$	Total number
* 3 weeks old	61	110	0	171
** E10.5 to 15.5	17	33	0	50



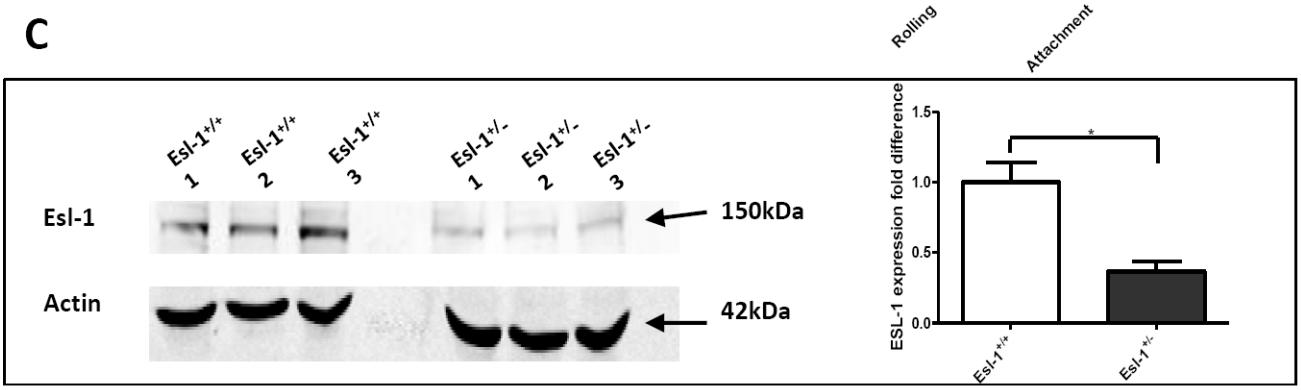


Figure 1. Characterization of ESL-1 deficient mice

A) Schematic representation of the wild-type and gene-trapped ESL-1 locus. B) Genotypes of offspring from matings of *ESL-1*$^{+/-}$ mice, * x^2=49.05, *p*=3.73E-13; ** x^2=14.25, *p*=0.00016. C) Western blot analysis of ESL-1 expression in spleen tissue (n=3 mice per group). *P*=0.015. D) Leukocyte-endothelial interactions in cremaster venules (n=5 mice per group), white bar represents *ESL-1*$^{+/+}$ mice and filled bar represents *ESL-1*$^{+/-}$ mice. * *p*=0.012, ***p*=0.002.

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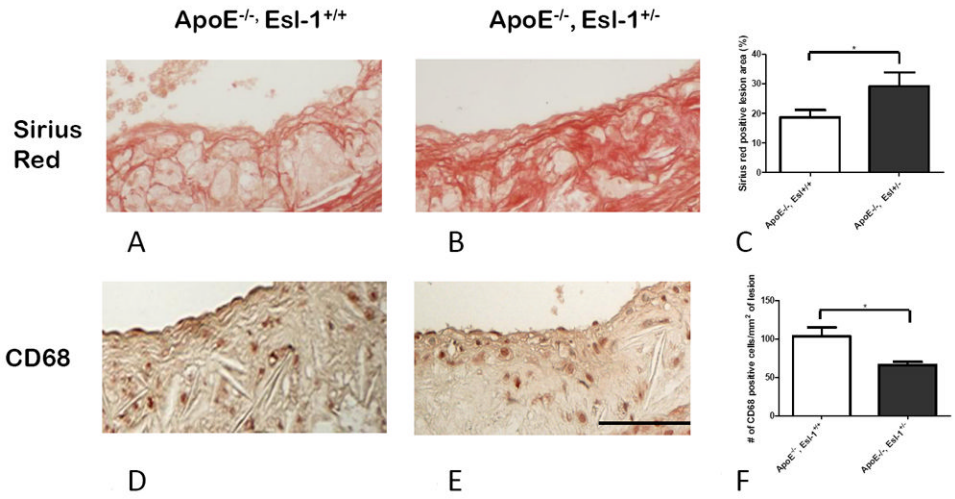

Figure 2. Effect of ESL-1 deficiency

Collagen specific Sirius Red staining of aortic valves by A) *ApoE*$^{-/-}$*,ESL-1*$^{+/+}$. B) *ApoE*$^{-/-}$*,ESL-1*$^{+/-}$. C) % of Sirius Red positive area of *ApoE*$^{-/-}$*,ESL-1*$^{+/+}$ (white bar) or *ApoE*$^{-/-}$*,ESL-1*$^{+/-}$ (filled bar) mice. p=0.043. Macrophage specific CD68 staining of aortic valves in D) *ApoE*$^{-/-}$*,ESL-1*$^{+/+}$. E) *ApoE*$^{-/-}$*,ESL-1*$^{+/-}$ F) Number of CD68 positive cells per mm^2 of lesion from *ApoE*$^{-/-}$*,ESL-1*$^{+/+}$ (white bar) or *ApoE*$^{-/-}$*,ESL-1*$^{+/-}$ (filled bar) mice. p=0.009. Scale bar=100μm.