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Distinct effects of nuclear membrane localization on gene transcription silencing in *Drosophila* S2 cells and germ cells

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Abstract

Nuclear envelope proteins have important roles in chromatin organization and signal-dependent transcriptional regulation. A previous study reported that the inner nuclear membrane protein, Otefin (Ote), was essential for germline stem cell (GSC) maintenance *via* interaction with Smad complex. The interaction of Ote with the Smad complex recruits the *bam* locus to the nuclear periphery and subsequently results in *bam* transcriptional silencing, revealing that nuclear peripheral localization is essential for *bam* gene regulation. However, it remains unknown whether the nuclear peripheral localization is sufficient for *bam* silencing. To address this issue, we have established a tethering system, in which the Gal4 DNA binding domain (DBD) of the Flag:Gal4 DBD:Ote△LEM fusion protein physically interacts with the Gal4 binding sites upstream of *bamP-gfp* to artificially recruit the reporter gene *gfp* to the nuclear membrane. Our data demonstrated that the nuclear peripheral localization seemed to affect the expression of the target naked gene in S2 cells. By contrast, in *Drosophila* germ cells, the nuclear membrane localization was not sufficient for gene silencing.

Keywords: Nuclear membrane proteins; Nuclear membrane localization; Gene transcription silencing; *Drosophila*

1. Introduction

The nuclear membrane separates the nucleus and the cytoplasm, and is composed of outer and inner nuclear membrane, the nuclear pore complex (NPC) and the lamina (Gruenbaum et al., 2005). The lamina is a meshwork of filament proteins between the nuclear periphery and the inner chromatin, and is located in the inner nuclear membrane. It is composed of lamin and lamin associated proteins, which are mainly nuclear membrane proteins, including Emerin, LAP2, Man1 and Otefin (Dechat et al., 2000; Gruenbaum et al., 2000; Goldman et al., 2002; Holaska et al., 2002). The lamina has important roles in multiple cell processes, including the maintenance of nuclear membrane morphology (Liu et al., 2000; Schirmer et al., 2001). It also provides the docking site for chromatins during the nuclear disassembly and reassembly in mitosis (Cohen et al., 2002), regulates DNA replication and transcription (Ellis et al., 1997; Spann et al., 1997; Izumi et al., 2000), and is involved in cell cycle regulation and chromosome segregation (Liu et al., 2000).

Drosophila ovarian germline stem cells (GSCs) provide an excellent system for studying fate determination of stem cells. Previous genetic analyses have identified a number of genes involved in the regulation of stem cells (Gregory et al., 2008; Tajbakhsh et al., 2009; Pera and Tam, 2010). Among them, one gene, the *bag-of-marbles* (*bam*) gene, which encodes a GSC/cystoblast (Cb) differentiation-promoting factor, has been shown to have a role in *Drosophila* Cb differentiation (McKearin and Ohlstein, 1995; McKearin, 1997). BMP ligands maintain GSCs by suppressing Cbs differentiation, which is achieved by silencing the transcription of the *bam* gene (Song et al., 2004). Another study showed that Otefin (Ote), the inner nuclear membrane protein in *Drosophila*, was both necessary and sufficient for GSC maintenance in *Drosophila*, which was affected by silencing *bam* transcription *via* interaction with Dpp signaling (Jiang et al., 2008). Furthermore, it has also been

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shown that the nuclear membrane localization of Ote is critical for its function in the maintenance of GSCs. However, it remains unknown whether Ote's nuclear peripheral localization is sufficient for *bam* silencing.

Ote is a member of the 'LEM' family, which represents an important group of nuclear membrane-associated proteins that have a conserved LEM domain. Previous studies demonstrated that Ote physically interacts with lamin B and YA proteins and localized at the nuclear envelope (Ashery-Padan et al., 1997a,b; Goldberg et al., 1998). The full length of *ote* gene is 1272 bp long and encodes a protein of 424 amino acids, containing a 50 amino acid LEM domain at the N terminus. The LEM domain interacts with barrier-to-auto integration (BAF), which binds DNA without any detectable sequence specificity. The transmembrane domain (TM) is located at the C terminus (amino acids 401−423), and is important for Ote's physical function of maintaining the self-renewal of GSCs (Jiang et al., 2008).

In order to explore the relationship between nuclear membrane localization and gene silencing, here we established a tethering system by integrating *ote* and the *bam* transcription reporter gene *bamP-gfp* into *Drosophila* chromatin respectively to analyze the function of nuclear membrane localization on gene transcription in germ cells. Our data showed that nuclear peripheral localization had an apparent influence on naked gene transcription and could lead to transcription silencing of the reporter gene in S2 cells. However, in *Drosophila* germ cells, nuclear peripheral localization was not sufficient to silence the transcription of the reporter gene.

2. Materials and methods

2.1. Constructs

2.1.1. Construct oteP-Flag-Gal4 DBD-Ote (50−400)

Flag, Gal4 DNA binding domain (DBD), and Ote (from 50 to 400 amino acids, without the TM domain and the LEM domain, △TM △LEM) were cloned into the plasmid under the control of the *ote* promoter (Jiang et al., 2008).

2.1.2. Construct oteP-Flag-Gal4 DBD-Ote (50−424)

Flag, Gal4 DNA binding domain (DBD), and Ote (from 50 to 424 amino acids, with the TM domain but without the LEM domain, △LEM) were cloned into the plasmid under the control of the *ote* promoter (Jiang et al., 2008).

2.1.3. UASP-Gal4 binding site-bamP-gfp

Two hundred and fifty-six copies of Gal4 binding site and *gfp* were cloned into UASP under the control of the *bam* promoter (from −800 to +133).

2.2. Drosophila genetics

Fly stocks used in this study were maintained under standard culture conditions. The following strains were used: (1) P {*oteP-Flag-Gal4 DBD-Ote* (*50−424*)*/cyo; △86/TM3*}, an *ote* allele with the TM domain but without the LEM domain; (2) P {*oteP-Flag-Gal4 DBD-Ote* (*50−400*)*/cyo; △86/TM3*}, an *ote* allele without the TM domain and the LEM domain; (3) P {*Gal4 binding site-bamP-gfp/cyo; △86/TM3*}. The transgenic flies P {*oteP-Flag-Gal4 DBD-Ote* (*50−424*)*/cyo; △86/TM3*} and P {*oteP-Flag-Gal4 DBD-Ote* (*50−400*)*/cyo; △86/TM3*} were single crossed with P {*Gal4 binding site-bamP-gfp/cyo; △86/TM3*} respectively at 25 °C for 10 days. The flies P {*Gal4 binding site-bamP-gfp/oteP-Flag-Gal4 DBD-Ote* (*50−424*)} were screened and prepared for the immunohistochemical analysis.

2.3. Immunohistochemistry

Ovaries were prepared for immunohistochemistry as described previously (Chen and McKearin, 2005). The following primary antibody dilutions were used: rabbit-anti-Ote (1:2000), rabbit-anti-Vasa (1:100, Santa Cruz, USA), rabbit-anti-GFP (1:5000, Invitrogen, USA), and mouse-anti-Flag (1:2000, Invitrogen). The following secondary antibodies were used at a 1:1000 dilution: goat-anti-mouse 555 (Invitrogen), goat-anti-rabbit Alexa 488 (Invitrogen). All samples were examined using a Zeiss microscope, and images were captured using the Zeiss Two-Photon Confocal LSM510 META system. Images were further processed with Adobe Photoshop 6.0.

2.4. Cell culture and transfection

S2 cells were cultured in Schneider's *Drosophila* medium (Sigma, USA). DNA construct transfection was performed using the Calcium Phosphate Transfection Kit (Specialty Media, USA) according to the manufacturer's instructions.

3. Results

3.1. The establishment of the tethering system for nuclear membrane localization

To explore whether nuclear membrane localization is sufficient for gene silencing, we designed a tethering system to recruit the target gene onto the nuclear membrane. As shown in Fig. 1, the target vector, UASP-*Gal4 binding site-bamP-gfp*, comprised of 256 copies of the Gal4 binding site, the *bam* promoter (from −800 to +133) and a reporter gene, *gfp*. The tethering vector, plasmid *oteP-Flag-Gal4 DBD-Ote* (*50−424*), contained the bait fusion protein comprising Ote (with the TM domain but without the LEM domain), Flag, and the Gal4 DBD, driven by *ote* promoter. Finally, as a control, we also constructed the random vector, plasmid *oteP-Flag-Gal4 DBD-Ote* (*50−400*), which encoded a fusion protein containing Ote without the TM domain, Flag, and the Gal4 DBD.

We hypothesized that if nuclear membrane localization was sufficient for gene silencing, then the fusion protein containing Ote (with the TM domain), Flag and Gla4 would be localized on the nuclear membrane, and subsequently, the reporter target vector, UASP-*Gal4 binding site-bamP-gfp* with multiple copies of Gal4 binding sites would be tethered onto the nuclear periphery membrane through Gal4 binding. Transcriptional

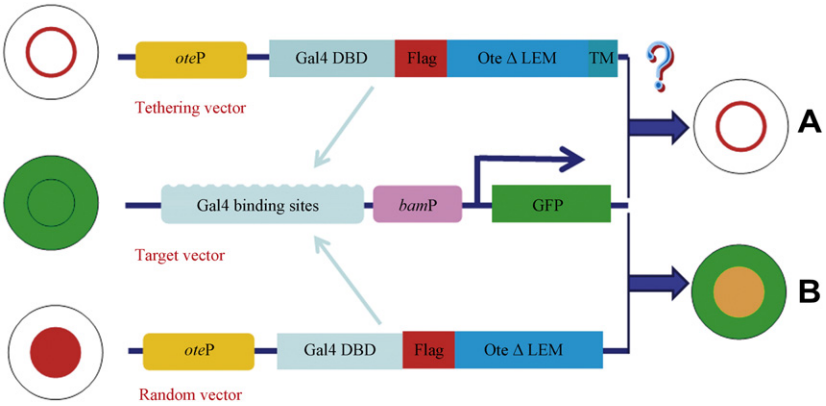

Fig. 1. Schematic illustration of the tethering system. The target vector contains 256 copies of the Gal4 binding site, *gfp*, and *bam* promoter. The tethering vector encodes a fusion protein of Gal4 DBD, Flag, and Ote (with the TM domain). The control vector encodes a fusion protein of Gal4 DBD, Flag, and Ote (without the TM domain). In S2 cells transfected with the target vector, the reporter gene *gfp* expresses both in the nucleus and the cytoplasm under the control of the *bam* promoter. In S2 cells co-transfected with the tethering vector and the target vector, the reporter gene *gfp* is tethered onto the peripheral membrane by the interaction between the Gal4 DBD and the Gal4 binding sites (**A**), leading to gene silencing. However, in S2 cells transfected with the random vector and the target vector, Ote is expressed in the nucleoplasm and can not facilitate bamP-GFP localization on the nuclear membrane (**B**).

regulation could be easily monitored by GFP fluorescence intensity (Fig. 1A). The negative control vector without the Ote TM domain should be primarily localized to the nucleoplasm, and there would be no tethering of the reporter gene to the nuclear peripheral region (Fig. 1B).

3.2. Nuclear membrane localization significantly repressed transcription of the gfp reporter gene in S2 cells

Firstly we tested the tethering system in S2 cells. In the S2 cells transfected with *oteP-Flag-Gal4 DBD-Ote* (*50−424*), the fusion protein was primarily localized on the nuclear membrane (Fig. 2A−C). However, in S2 cells transfected with *oteP-Flag-Gal4 DBD-Ote* (*50−400*), the exogenously produced fusion protein was primarily localized in the nucleoplasm (Fig. 2D and F). S2 cells transfected by plasmid *Gal4 binding site-bamP-gfp* and stained with anti-GFP showed that GFP protein distributed in both the nuclear and the cytoplasm regions (Fig. 2G).

In S2 cells co-transfected with *oteP-Flag-Gal4 DBD-Ote* (*50−424*) and *bamP-Gal4 binding site-gfp*, the GFP expression level was significantly reduced (Fig. 3A−D). Similar results were observed using real-time PCR technology. The expression of *gfp* mRNA was reduced in cells co-transfected with the tethering vector and the target vector compared with cells co-transfected with the random plasmid vector and the target vector (Fig. 3I). However, the co-expression of *oteP-Flag-Gal4 DBD-Ote* (*50−400*) and the reporting target vector

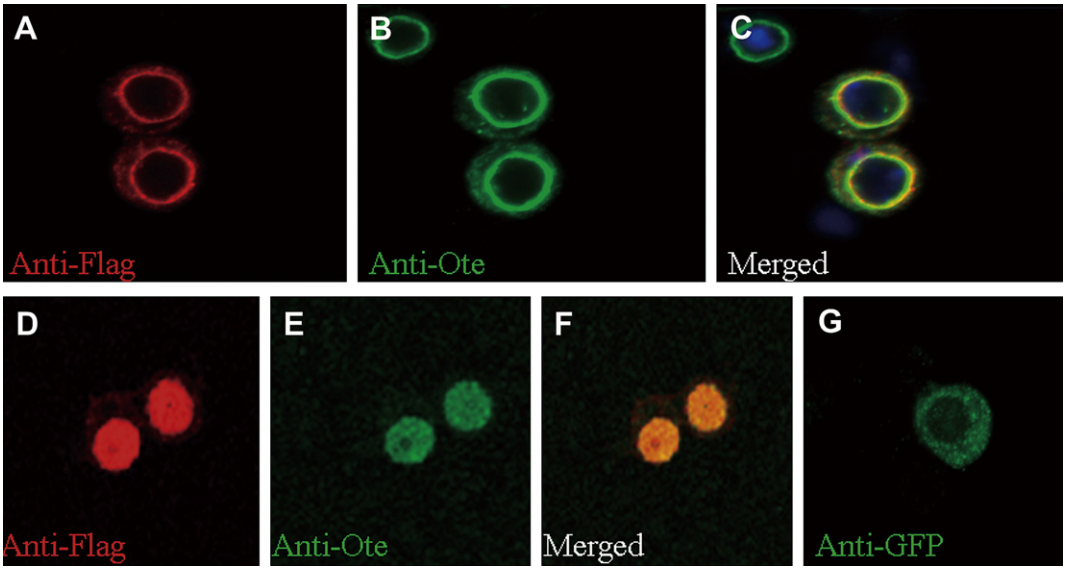

Fig. 2. Identification of the expression pattern of Ote. S2 cells were stained with anti-Flag (red) to visualize the localization of the Flag-Ote fusion protein, and with anti-Ote (green) to outline both the endogenous and the exogenous Ote. **A:** Ote with the TM domain localized at the nuclear membrane. **B:** both the endogenous and the exogenous Ote localized at the nuclear membrane. **C:** the merged image of **A** and **B**. **D:** Ote (△TM △LEM) localized in the nucleoplasm instead of the nuclear membrane. **E:** the endogenous Ote localized at the nuclear membrane while the exogenous Ote without the TM localized in the nucleoplasm. **F:** the merged image of **D** and **E**. **G:** S2 cells transfected by plasmid *Gal4 binding site-bamP-gfp* were stained by anti-GFP, showing the expression pattern of the reporter gene *bamP-gfp*.

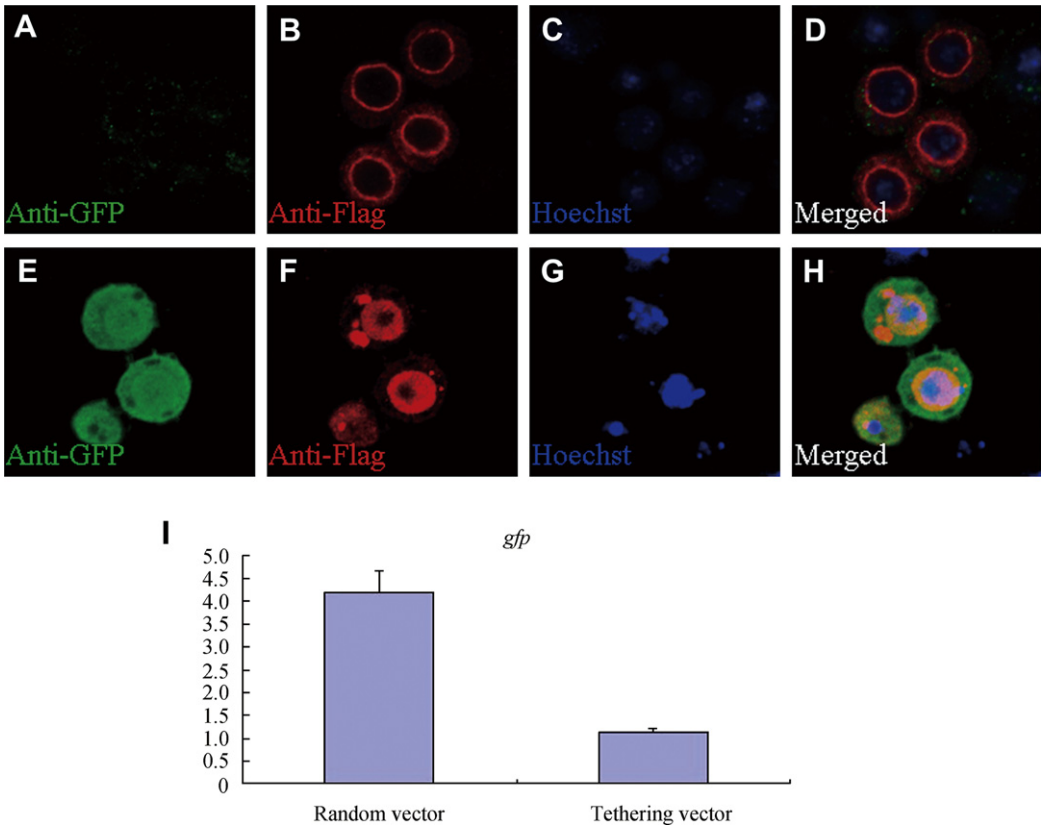

Fig. 3. Nuclear membrane-localized Ote influenced the expression of *bamP-gfp* in the S2 cell system. In S2 cells co-transfected with the tethering vector and the target vector, S2 cells stained by anti-GFP showed that the expression of the transcription reporter gene *bamP-gfp* was sharply reduced in contrast with the situation in cells co-transfected with the random vector and the target vector (**A**). **B:** S2 cells stained by anti-Flag to show the localization of exogenous Ote. **C:** S2 cells stained by Hoechst to show the nuclei. **D:** merged image showing that the exogenous Ote localized on the nuclear membrane. In S2 cells co-transfected with the random vector and the target vector, S2 cells stained by anti-GFP showing that the expression of reporter gene *bamP-gfp* did not significantly change (**E**). **F:** S2 cells were stained by anti-Flag to show the localization of exogenous Ote. **G:** S2 cells stained by Hoechst to show the nuclei. **H:** merged image showing that the exogenous Ote localized in the nucleoplasm and did not repress the expression of *bamP-gfp*. **I:** one group of S2 cells were transfected with the random vector, target vector and pAC5.1-lacZ, the other group of S2 cells were transfected with the tethering vector, target vector and pAC5.1-lacZ. LacZ served as a transfection efficiency control. After 36 h post transfection, an RT-PCR assay was performed. The *gfp* mRNA level of cells transfected with the tethering vector and target vector was obviously reduced compared with that of cells transfected with the random vector and target vector.

did not perturb the protein expression pattern of GFP (Fig. 3E−H). These results indicated that the tethering system worked in S2 cells.

3.3. Nuclear membrane localization did not significantly influence gene expression in Drosophila germ cells in vivo

Based on the results in S2 cells, we wondered whether this tethering system was also effective in *Drosophila* germ cells under native physiological conditions. We tested this hypothesis by making transgenic fly stocks. The expression pattern of reporter gene *bamP-gfp* in the germ cells of fly P {*Gal4 binding site-bamP-gfp}* was consistent with that of native *bam* gene, which was expressed at the rear region of the germarium, but not in GSCs and cystoblast cells (Fig. 4). The exogenously expressed Ote with the TM domain was primarily localized on the nuclear membrane in the fly P {*oteP-Flag-Gal4 DBD-Ote* (*50−424*)} (Fig. 5).

After a single cross of fly P {*Gal4 binding site-bamP-gfp*} with fly P {*oteP-Flag-Gal4 DBD-Ote* (*50−424*)}, we screened for the P {*Gal4 binding site-bamP-gfp/oteP-Flag-Gal4 DBD-Ote* (*50−424*)} flies. In the germarium of these flies, we hypothesized that the reporter gene *bamP-gfp* would be tethered onto the nuclear periphery. We examined the expression level of GFP in the germ cells of these flies by immunohistochemistry. The expression pattern of GFP remained similar to the expression pattern in stem cells of P {*Gal4 binding site-bamP-gfp*}, and the protein expression level of GFP was not affected by the nuclear membrane localization of *oteP-Flag-Gal4 DBD-Ote* (*50−424*) (Fig. 6).

Thus, we concluded that our tethering system, unlike the case in S2 cells, does not work in the germ cells of *Drosophila* ovaries. Further experiments are required to account for this discrepancy.

4. Discussion

The non-random distribution of chromatin in the nucleus implies that the sub compartment of the nucleus has specific roles in chromatin organization and transcriptional regulation

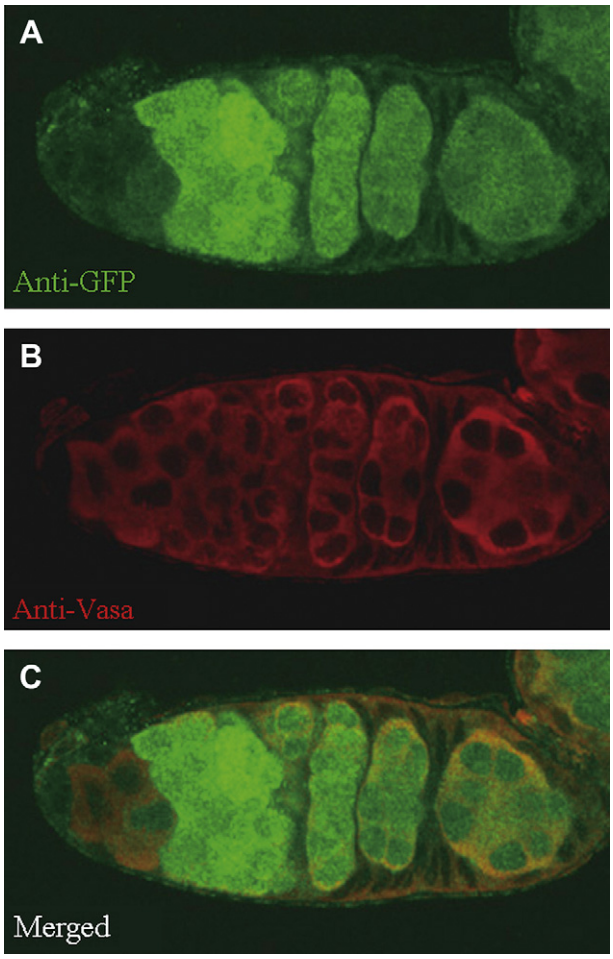

Fig. 4. The expression pattern of GFP in the germ cells of P {*Gal4 binding site-bamP-gfp*}. **A:** the ovary of transgenic fly P {*Gal4 binding site-bamP-gfp*} stained by anti-GFP to show the expression pattern of GFP. The expression pattern of GFP was the same as that of the wild type *bam* gene. **B:** the same ovary stained by anti-Vasa to outline the germ cells in the germarium of *Drosophila*. **C:** merged image of the *Drosophila* germarium stained by anti-GFP and anti-Vasa.

(Ahmed and Brickner, 2007). Research in budding yeast showed that the repressed region of the genome closely interacts with the nuclear periphery and the inactive chromatin regions, such as telomeres and mating-type loci, are connected to the nuclear membrane. Nuclear membrane localization promotes gene silencing near the telomere. On the other hand, recruiting the mating-type loci with defective silencing elements to the nuclear periphery restored its silencing (Andrulis et al., 1998). However, it is not clear whether the nuclear membrane localization is the result of or the reason for gene silencing. The tethering system was developed to answer this question. Many tethering experiments in mammalian cells have recruited certain genes to the inner nuclear membrane to analyze the impact of nuclear membrane localization on gene transcription. It has been found that most of the genes were resistant to the repressive role of the nuclear periphery; however, the transcription of some genes could be silenced (Dillon, 2008). Chromatin immune precipitation based on the scale of yeast genome has shown that numerous active genes physically interacted with the components of the nuclear pore complex (NPC) (Casolari et al., 2004, 2005), among which Nup2 played an active role in blocking the spread of the heterochromatin region. Nup2 probably protected these sites from the impact of the adjacent heterochromatin (Burgess-Beusse et al., 2002; Ishii et al., 2002; Dilworth et al., 2005). In addition, it has been found that the transcriptionally active yeast genes interacted with the SAGA complex of the NPC, which was supposed to have functions in mRNA transportation (Kurshakova et al., 2007). It seems that the nuclear periphery, except for the NPC, forms a repressive environment, while the region near the NPC forms an active environment for gene transcription.

The *Drosophila* ovarian GSCs within the germarium region provide a good system for studying stem cell self renewal and differentiation. GSCs normally divide asymmetrically to ensure self-renewal and differentiation. During this process, several mechanisms are required, including the BMP/Dpp pathway (Xie and Spradling, 1998; Chen and McKearin, 2003; Song et al., 2004), miRNA-mediated pathways (Forstemann et al., 2005; Hatfield et al., 2005; Jin and Xie, 2007; Park et al., 2007; Yang et al., 2007) and the ubiquitin-mediated cyclinA degradation pathway (Chen et al., 2009). Furthermore, nanos and pumilio-mediated translation pathways are also involved (Lin and Spradling, 1997; Forbes and Lehmann, 1998). Recent research has shown that Fused (Fu), a serine/threonine kinase that regulates Hedgehog, functions in concert with the E3 ligase Smurf to regulate ubiquitination and proteolysis of the BMP receptor Thickveins in Cbs. This regulation generates a steep gradient of BMP activity between GSCs and Cbs, allowing for bam expression on Cbs and concomitant differentiation (Xia et al., 2010).

Previous research indicated that Ote, the inner nuclear membrane protein in *Drosophila*, was both necessary and sufficient for GSC maintenance (Jiang et al., 2008). Ote is required for the Dpp/BMP signaling pathway-mediated silencing of *bam* transcription, and the nuclear membrane localization of Ote is essential for its role in GSC maintenance (Jiang et al., 2008). In the present study, *ote* and the *bam* transcription reporter gene *bamP-gfp* were integrated into the chromosome of *Drosophila*. When the transgenic fly P {*Gal4 binding site-bamP-gfp*} was crossed with P {*oteP-Flag-Gal4 BD-Ote*(*50–424*)}, the chromosome with the integration of *Gal4 binding site-bamP-gfp* was tethered onto the nuclear membrane. Chromosomes in the nuclear periphery are subject to histone modification, such as deacetylation and methylation, which influences gene conformation and expression. When chromatin is highly condensed to form heterochromatin, genes are silenced. It is possible that, because of complex physical conditions, such as histone modification, the multiple copies of transgene near the nuclear periphery form a microenvironment to counteract the repressive effect of nuclear membrane localization on *bamP-gfp* expression in germ cells of *Drosophila*. Furthermore, *ote* interacts with *bam* indirectly; therefore, the reporter gene may escape from the lamina's repressive effect. We propose that it is possible that the large number of copies of an exogenous gene could create a micro-environment to offset the repressive role of the nuclear lamina.

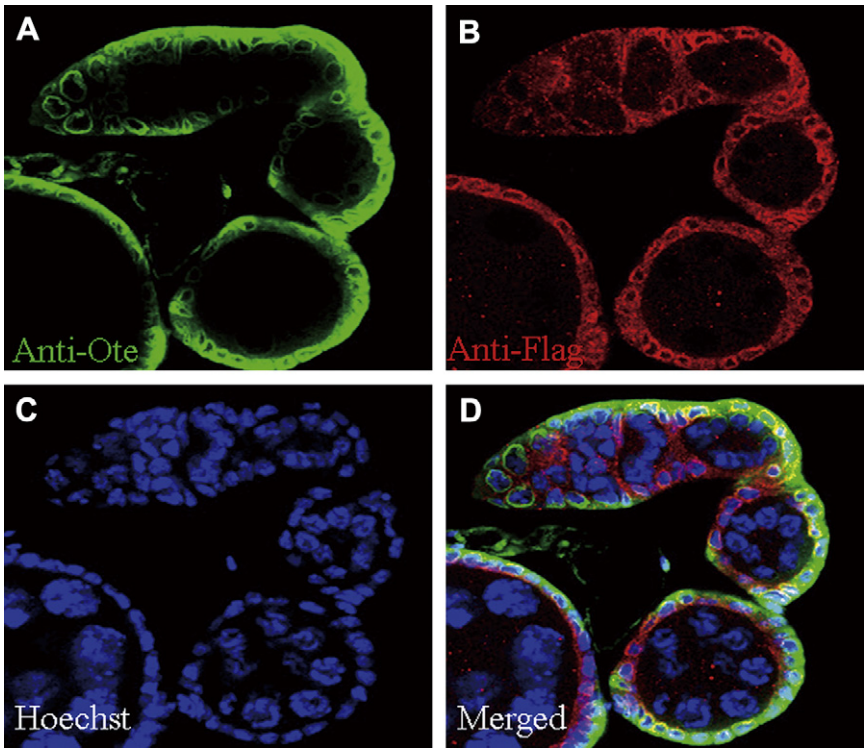

Fig. 5. The expression pattern of exogenous Ote in transgenic fly P {*oteP-Flag-Gal4 DBD-Ote* (*50−424*)}. **A:** the ovary of transgenetic fly P {*oteP-Flag-Gal4 BD-Ote* (*50−424*)}stained by anti-Ote to show both the endogenous and exogenous Ote. **B:** the same ovary stained by anti-Flag to show the localization of exogenous Ote, which was on the nuclear membrane. **C:** *Drosophila* cells stained by Hoechst to show the nuclei. **D:** merged picture of the *Drosophila* germarium stained by anti-Ote, anti-Flag and Hoechst.

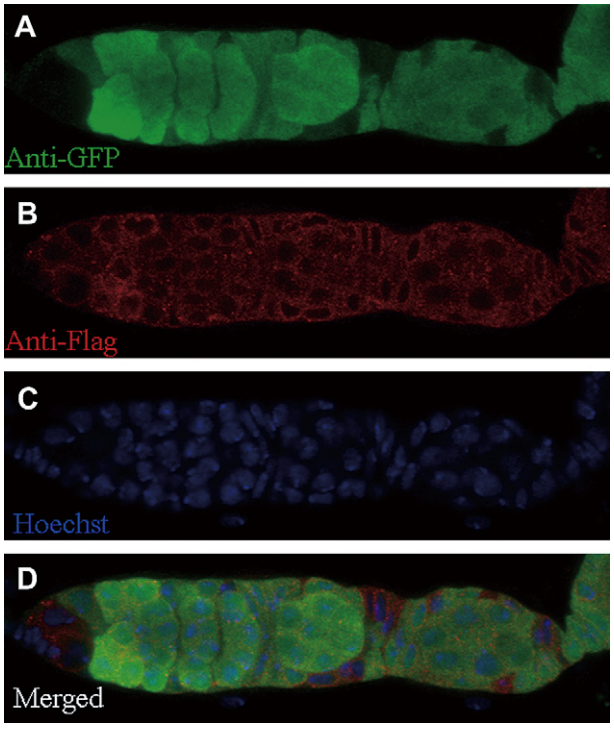

Fig. 6. The expression of GFP in germ cells of fly P {*Gal4 binding site-bamP-gfp*/*oteP-Flag-Gal4 DBD-Ote* (*50−424*)}. **A:** the ovary stained by anti-GFP to show the expression of GFP. The expression pattern did not significantly change compared with that of fly P {*Gal4 binding site-bamP-gfp*}. **B:** the same ovary stained by anti-Flag to show the exogenous Ote. **C:** *Drosophila* cells stained by Hoechst to show the nuclei. **D:** merged image of the *Drosophila* germarium stained by anti-GFP and anti-Flag.

Using tethering methods, it has also been illustrated that, in mammalian cells, most mammalian genes are resistant to the repressive role of the nuclear periphery. Only a small group of genes could be repressed. This confirms the view that the nuclear lamina is not generally a refractory environment for gene expression (Dillon, 2008). In S2 cells, the situation is simple. Cells are transiently transformed with plasmids, and the naked DNA is tethered to the nuclear periphery, where nuclear membrane localization is sufficient to silence *bamP-gfp*.

In conclusion, nuclear membrane localization had an influence on naked gene transcription and could lead to gene silencing of *bamP-gfp* in the S2 cell culture system. By contrast, in germ cells of *Drosophila* this impact was not apparent and the nuclear membrane localization was not sufficient to silence gene transcription.

Acknowledgements

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