Treadmill running induces satellite cell activation in diabetic mice

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A B S T R A C T
Skeletal muscle-derived stem cells, termed as satellite cells, play essential roles in regeneration after muscle injury in adult skeletal muscle. Diabetes mellitus (DM), one of the most common metabolic diseases, causes impairments of satellite cell function. However, the studies of the countermeasures for the DM-induced dysfunction of satellite cells have been poor. Here, we investigated the effects of chronic running exercise on satellite cell activation in diabetic mice focused on the molecular mechanism including Notch and Wnt signaling, which are contribute to the fate determination of satellite cells. Male C57BL/6 mice 4 weeks of age were injected with streptozotocin and were randomly divided into runner group and control group. Runner group mice were performed treadmill running for 4 weeks. DM attenuated satellite cell activation and the expressions of the components of Notch and Wnt signaling. However, chronic running resulted in activation of satellite cells in diabetic mice and salvaged the inactivity of Wnt signaling but not Notch signaling. Our results suggest that chronic running induces satellite cell activation via upregulation of Wnt signaling in diabetic as well as normal mice.

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1. Introduction
Muscle-specific stem cells, termed as satellite cells, are located between the basal lamina and plasma membrane of muscle fibers [1]. Satellite cells are mitotically quiescent under normal physiological conditions but are activated in response to stimulation, such as muscle injury, and proliferate extensively. Majority of satellite cells differentiate into mature muscle fibers, whereas others return to a quiescent state to self-renew and maintain the stem cell pool. In adult skeletal muscle, satellite cells play an essential role in muscle regeneration and maintain the plasticity of the skeletal muscle [2,3].

Diabetes mellitus (DM) is one of the most common metabolic diseases worldwide, and the number of patients with DM increased in recent years. Patients with DM exhibit hyperglycemia caused by impairments in insulin secretion (type 1), or action (type 2), or both. Type 1 DM is characterized by an immune-mediated destruction of β cells in the pancreatic islets of Langerhans, leading to insulin deficiency [4]. It is well known that type 1 DM is developed in childhood and can lead to severe long-term complications including retinopathy, neuropathy and nephropathy [5]. On the other hand, type 2 DM occurs through mechanisms such as insulin resistance in peripheral tissues and increased blood glucose levels caused by overnutrition [6,7]. DM is often associated with the development of secondary complications in various organs, such as eyes, kidneys, heart, brain, and skeletal muscle [8]. Previous studies have reported that DM induces a variety of alterations in the structure and function of the skeletal muscle, such as muscle atrophy [9], fiber-type transition [10], muscle weakness [11], and a decline in energy metabolism [12]. In addition, DM attenuates satellite cell function, including proliferation, differentiation, and subsequent muscle regeneration. Satellite cells derived from diabetic mice have an impaired ability to differentiate into myotubes [13]. The delay of regeneration after muscle injury was observed in several models of DM such as Akita, ob/ob, and db/db mice [14,15]. Although the effects of DM on the satellite cell function have been extensively investigated, not many studies have focused on countermeasures for diabetes-induced attenuation of satellite cells, and the molecular mechanisms underlying the changes remain unclear.

One of the candidates for the countermeasure is physical exercise, which contributes to satellite cell activation and proliferation. The increments in the number and proliferative ability of satellite cells have been previously reported in both human and animal studies [16,17]. Physical exercise also induces the change in
extracellular signaling in the skeletal muscle that affects satellite cells. For instance, Notch signaling, which is involved in cell fate choice and regulates satellite cell proliferation, is activated by exercise according to increased expressions of ligands, Notch receptors, and downstream effectors in myogenic cells [18,19]. Conversely, exercise-induced upregulation of Wnt signaling, which contributes to satellite cell activation and lineage specification in the skeletal muscle, has been reported [20,21]. However, it remains unclear whether physical exercise can prevent satellite cell dysfunction, including the activities of Notch and Wnt signaling, by DM.

In the present study, we investigated the molecular mechanisms of satellite cell activation by chronic running exercise in diabetic mice with a focus on the Notch and Wnt signaling pathway. We used type 1 DM model mice generated by intraperitoneal administration of streptozotocin, which is a compound that displays a preferential toxicity toward pancreatic β cells. We found that DM decreased the number of satellite cells and inhibited satellite cell activation via downregulation of Notch and Wnt signalings. However, chronic running improved Wnt signaling activity, but not Notch signaling in diabetic mice. Of note, chronic running increased the number of activated satellite cell in diabetic as well as normal mice. Thus, these results may provide future perspectives on exercise-based medicine as a countermeasure for DM-induced dysfunction of satellite cells.

2. Materials and methods

2.1. Animals

Experiments were performed on 4-week-old male C57BL/6 mice (Japan SLC Inc., Hamamatsu, Japan) weighing 19–21 g. DM was induced by a single intraperitoneal injection of 200-mg/kg streptozotocin (STZ, Wako) dissolved in citrate buffer. The blood glucose levels were measured 1 week after injection, and mice with blood glucose levels higher than 300 mg/dL were considered diabetic as previously described [22]. Mice were randomly divided into four groups (n=5 per group): control (Cont), runner (Run), diabetes (DB), and diabetes/runner (DB+Run). Mice in the DB and DB+Run groups were injected with STZ, and mice in the Cont and Run groups were injected with an analogous volume of citrate buffer. Animals were housed in standard cages in facilities with controlled temperature and humidity under a 12:12-h light/dark cycle and had free access to chow and water. Animal experiments were performed in a humane manner after receiving approval from the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology.

2.2. Treadmill running and tissue sampling

Animals in the Run and DB+Run groups performed treadmill running (10–20 m/min, 60 min/day, 5 days/week) for 4 weeks starting 1 week after injection of citrate buffer or STZ. After 4 weeks of running, the mice were sacrificed by cervical dislocation. The plantaris (for biochemical analysis) and gastrocnemius (for immunohistochemistry) were dissected from each mouse and frozen in liquid nitrogen after measuring the wet weight and stored at −80°C until analysis.

2.3. Immunohistochemistry (IHC)

Cross-sections of the midportion of the gastrocnemius were cut at 10 µm in a cryostat (Microm Cryo-Star HM 560, Walldorf, Germany) and maintained at −20°C until analyses. The sections were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for 5 min and degreased in 100% methanol for 10 min at −20°C. After being washed by phosphate-buffer saline (PBS), the sections were blocked in 10% donkey serum diluted with PBS containing 0.1% Triton-X 100 (PBS-T) for 20 min. Then, the sections were incubated overnight at 4°C with anti-Laminin α2 (1:600; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and anti-MyoD (1:200; Santa Cruz Biotechnology) antibodies diluted in PBS-T containing 1% bovine serum albumin (BSA). Immunoreactivity was detected by incubation with Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and AlexaFluor 488-conjugated donkey anti-rabbit IgG (1:1500; Life Technologies, Carlsbad, CA, USA) diluted in PBS-T containing 1% BSA for 4 h. Sections were counterstained with 4',6-diamidino-2-phenylindole (Wako Pure Chemical Industries, Osaka, Japan). After several washes, the stained sections were mounted using the mounting medium (KPL, Gaithersburg, MD, USA). Images were acquired using an Olympus FV1000-D confocal microscope (Olympus, Tokyo, Japan).

2.4. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from frozen plantaris muscles using Isogen. RNA samples were treated with Turbo DNase to remove genomic DNA. cDNA synthesis was performed using PrimeScript RT Master Mix (Takara Bio., Otsu, Japan) according to the manufacturer’s recommendations. qRT-PCR analysis were performed as previously described [23] and specific primers obtained from Life Technologies (Table 1).

2.5. Protein extraction and Western blot analysis

Protein extraction from plantaris muscles and Western blot analysis were performed as previously described [23].

2.6. Statistical analysis

Data were analyzed using the Student’s t test and were expressed as mean ± SE. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. STZ-induced diabetes gives rise to skeletal muscle atrophy

Intraperitoneal injection of STZ resulted in the development of pronounced hyperglycemia in experimental animals [24]; the blood glucose levels of all mice in the DB and DB+Run groups were greater than 300 mg/dL 1 week after injection. In a previous study, STZ-induced diabetes resulted in severe muscle atrophy [25]. Accordingly, body weight and wet weights of the plantaris and gastrocnemius muscles of the DB group were significantly lower than that of the Cont group at the end of the experiment (Fig. 1A–C). Four weeks of treadmill running did not affect on the muscle weights in both normal and diabetic mice, as indicated by the unchanged wet weights of the plantaris and gastrocnemius muscles to body mass ratio in the trained animals compared with those of the sedentary animals (Fig. 1A–C). The cross-sectional areas of gastrocnemius muscles were also lower in diabetic mice (Fig. 1D, E) regardless of performing exercise. These data suggest that STZ-induced diabetes causes skeletal muscle atrophy and chronic running cannot salvage the phenotype.
3.2. STZ-induced diabetes attenuates but chronic running activates satellite cells

We examined the effects of diabetes on satellite cell activation and proliferation. Activated satellite cells express the myogenic regulatory factors Myf5 and MyoD, two key transcription factors for myogenic lineage progression and differentiation, in addition to the stem cell–specific transcription factor Pax7 [26]. To investigate changes in the number as well as the characteristics of satellite cells, we performed immunohistochemical analysis of Pax7 and MyoD using cross sections of the gastrocnemius muscle and evaluated the expression of these markers (Fig. 2A). The number of Pax7+ cells was slightly decreased in the DB group (Fig. 2B). We also examined the effects of chronic running as a countermeasure for diabetes–induced dysfunction of satellite cells. The number of Pax7+ cells significantly increased following 4-week treadmill running in both normal and diabetic mice (Fig. 2B). Because positive immunoreaction of Pax7 is observed in both quiescent and activated satellite cells, we quantified Pax7+ MyoD+ cells in the cross sections. Pax7+ MyoD+–activated satellite cells were rarely observed in DB mice but were prevalent in the DB+Run group; the number of Pax7+ MyoD+ cells were decreased by diabetes, whereas they significantly increased following chronic running in both normal and diabetic mice (Fig. 2C). Therefore, these results suggest that diabetes inhibits satellite cell activation and subsequent proliferation in the skeletal muscle, but chronic running salvages the dysfunction.

Because of the finding of an activated population within satellite cells during IHC analysis, we performed a Western blot analysis to confirm and compare the expression levels of the marker proteins of activated satellite cells Myf5 and MyoD using skeletal muscle–derived protein extract from each mouse. The expression levels of Myf5 and MyoD were reduced by diabetes, whereas they were markedly upregulated following chronic running in both normal and diabetic mice (Fig. 2D). Because Myf5 is only expressed in Pax7+ cells but not in myofibers and MyoD is also expressed in satellite cells or differentiating myoblasts [27], these data are reflected in the expression of Myf5 and MyoD in satellite cells and myoblasts in the skeletal muscle. These results are consistent with those of the IHC analysis and suggest that the changes in the expression of Myf5 and MyoD may contribute to the changes in the population within Pax7+ cells from the quiescent to activated states.

3.3. STZ-induced diabetes downregulates Notch signaling and chronic running cannot salvage the dysfunction

To examine the molecular mechanism underlying the changes in the number of satellite cells in relation to diabetes or chronic running, we focused on the Notch signaling pathway, which regulates satellite cell self-renewal and proliferation. Binding of Notch receptors to their ligands releases the Notch intracellular domain, which translocates into the nucleus and binds to the recombination signal binding protein for immunoglobulin kappa J, thereby promoting the transcription of target genes, such as those belonging to the Hes and Hey families [28,29]. Upregulation of Notch signaling promotes the transition of satellite cells to proliferative myogenic precursor cells and myoblast proliferation is decreased when the Notch activity is inhibited in myoblasts [30]. Using qRT-PCR, we assessed the expression of Delta like ligand 4 (DLL4), which is one of the ligands bound to the Notch receptors and Notch target genes, Hes1 and Heyl. The transcript levels were significantly lower in diabetic mice than in normal mice, and chronic running did not affect the expression of Notch signaling components (Fig. 3). These results suggest that STZ-induced diabetes induces downregulation of Notch activity, resulting in a decreased number of satellite cells.

3.4. STZ-induced diabetes downregulates Wnt signaling but chronic running salvages the dysfunction

We also investigated the Wnt signaling pathway, which regulates satellite cell activation and promotes myogenic differentiation, to examine the molecular mechanism underlying the changes in the characteristics of satellite cells by diabetes or chronic running. Wnts are secreted extracellular ligands that bind to Frizzled receptors (Fzd) in the plasma membrane [31], and stabilizes β-catenin, which forms a complex with the T cell factor (TCF)/leukocyte enhancer factor (LEF) that is translocated into the nucleus and activates the transcription of target genes [32,33]. Wnt signaling regulates myogenesis via modulation of the expression of Myf5 and MyoD [34], and it was recently reported that the Wnt signaling is upregulated after resistance or endurance exercise [23,35]. We investigated whether diabetes downregulates Wnt activity and chronic running salvages this dysfunction in the skeletal muscle. As expected, Wnt3, Wnt5a and Wnt5b mRNA levels were significantly decreased in the DB group, whereas the expressions of Wnt3 and Wnt5a were recovered following chronic running (Fig. 4A). These data were consistent with the changes in the number of satellite cells and the expressions of marker proteins.

Next, we evaluated intracellular signal transduction because the Wnt member proteins obviously regulate the satellite cell activation under diabetic conditions. The protein expression of β-catenin was investigated by Western blot analysis. B-catenin, which is the intracellular signal transducer of Wnt signaling and acts in the nucleus with TCF and LEF transcriptional factors, is phosphorylated by Glycogen synthase kinase (GSK)-3β, which leads to its ubiquitin-dependent degradation in the absence of Wnt ligands [32]. However, when Wnt binds to Fzd, GSK-3β is phosphorylated (inactivated), and β-catenin is stabilized and translocated into the nucleus to activate transcription of target genes. The level of β-catenin decreased in the DB group, but this protein accumulated by chronic running (Fig. 4B). These results regarding the expression of the Wnt gene and the level of β-catenin suggest that diabetes attenuated Wnt activity, whereas chronic running salvaged the dysfunction in the skeletal muscle.

4. Discussion

The present study investigated the effects of chronic running as the countermeasure for diabetes–induced dysfunction of satellite cells and the molecular mechanism underlying the changes. Our analysis showed that diabetes decreases the number of satellite

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**Table 1**

| Table 1 | Primer sequences for qRT-PCR. | | |
| --- | --- | --- | --- |
| **Target gene** | **Sequence (5'-3')** | **Product length** |
| GAPDH | Forward: TCTGCTGTCGGACTCTACTG | 157 bp |
| | Reverse: CCGAGGCACTTGGTCGATTC | |
| DLL4 | Forward: ACAAGAATCCGCGGACGTTGCCA | 179 bp |
| | Reverse: ACCACAGCAAGAGAAGCTGTGGATG | |
| Hes1 | Forward: TGCTTTCTCATCCCAAAAC | 137 bp |
| | Reverse: ACGTGAGTCCGCAAGTACC | |
| HeyL | Forward: GCGACACAGTCCTTGTGATC | 165 bp |
| | Reverse: TGCATAGCCTTGGAGTGGG | |
| Wnt3 | Forward: CCAACACACACACAGCGAAGAAC | 151 bp |
| | Reverse: CCGCACATTACACACCCCTTGGAG | |
| Wnt5a | Forward: TGTACCTGCCGGAGCTTGGC | 164 bp |
| | Reverse: TCGACACTCACGGCTACACTGC | |
| Wnt5b | Forward: CAGGGCAATGGATGGTGGGAG | 185 bp |
| | Reverse: AGGAATTGGGTCGCACACGG | |
cells and inhibits satellite cell activation via downregulation of Notch and Wnt signaling, whereas chronic running improves Wnt activity and salvages satellite cell activation in diabetic mice.

DM has been reported to decrease in the regenerative ability of satellite cells in the skeletal muscle [14,15]; however, the change in satellite cell characteristics under diabetic conditions and the mechanistic basis for the attenuation of satellite cell function have not been reported. Thus, we examined the molecular mechanism underlying diabetes-induced satellite cell dysfunction focusing on modulation of Notch and Wnt signalings, which contribute to the fate choice in the satellite cells. We determined the gene expressions of Notch and Wnt signaling components in whole plantaris muscles. Since a previous study has demonstrated that HeyL, which is one of the Notch target genes, is exclusively expressed in satellite cells in skeletal muscle [36], the expression of HeyL in the present study, reflected the expression in satellite cells. Hes1, on the other hand, is expressed in not only satellite cells but also non-satellite cells in skeletal muscle. Thus, we cannot exclude the possibility that Hes1 data in the present study may include the non-satellite cells expression profile. Further studies using flow cytometer to directly isolate satellite cells are crucial for correctly understanding the effects of DM or exercise on the activity of Notch signaling in satellite cells.

Our immunohistochemical analysis showed that the number of Pax7⁺/MyoD⁺-activated satellite cells was decreased in diabetic mice, suggesting that DM inhibits satellite cell activation in the skeletal muscle. Because previous studies have shown that Notch signaling plays a critical role in maintenance of the quiescent satellite cell state [37,38], we expected that Notch signaling was activated in diabetic mice. However, in the present study, the gene expressions of Notch signaling components DLL4, Hes1, and HeyL were decreased by STZ-induced diabetes. The discrepancy between the inhibition of satellite cell activation and the downregulation of Notch signaling in the present study may be explained by regulation of MyoD, which is a key transcription factor for myogenic lineage progression and differentiation. Hes and Hey family of Notch target genes are known to form a heterodimer with MyoD to block its function [39,40]. Therefore, we hypothesize that Wnt signaling regulates MyoD transcription before Notch activation in satellite cells. In the present study, Wnt signaling was downregulated in diabetic mice according to decreased gene expressions of Wnt3, Wnt5a, and Wnt5b and β-catenin stabilization. A previous study demonstrated that hyperglycemia induced by an intake of a high-fat diet results in decreased levels of Wnt1, Wnt3a, and Wnt5a mRNA and β-catenin

Fig. 1. Skeletal muscle atrophy by STZ-induced diabetes. A. Change in body weight caused by diabetes. A graph representing the mean of body weight of each group. B. C. Change in muscle weights caused by diabetes. Graphs representing wet weights of plantaris muscle (B) and gastrocnemius muscle (C) normalized to the body weights in each group at the end of the experiment. D. E. Change in cross-sectional area (CSA) of muscle fibers caused by diabetes. Representative images of immunohistochemistry staining for Laminin using gastrocnemius sections from Cont and DB groups (D) and a graph plotting the means of CSA in each group (E) are shown. All values are expressed as the mean ± SEM (n = 5). Significant differences: *compared to control group (P < 0.05).
Fig. 2. Satellite cell activation following chronic running salvaging diabetes-induced attenuation. A. B. C. Immunohistochemistry analysis of cross-section of gastrocnemius muscles. Representative merged images of immunohistochemistry staining for Pax7 (green) and MyoD (red) with DAPI from each group are shown (A). Magnification of the area surrounded by the dotted square is shown in the right panels. The proportions of Pax7(+) cells per total myonuclei (B) and that of Pax7(+)MyoD(+) cells per total Pax7(+) cells (C) are shown. White arrows and arrowheads indicate Pax7(+) cells and Pax7(+)MyoD(+) cells, respectively. D. Expression profiles of marker proteins of activated satellite cells. The protein expression levels of Myf5 and MyoD were detected by Western blot. The right images represent the typical blot patterns of Myf5, MyoD, and GAPDH. All values are expressed as the mean ± SEM (n=5). Significant differences: *compared to control group (P < 0.05), #compared to DB group (P < 0.05).
Fig. 3. Downregulation of Notch signaling pathway by diabetes and chronic running cannot salvage these changes. Expression levels of Notch signaling-related genes. Amounts of DLL4, Hes1, and HeyL mRNAs in the plantaris were measured by qRT-PCR analysis. Target mRNA expressions were normalized to that of GAPDH and then plotted as the expression ratio relative to control group. All values are expressed as the mean ± SEM (n=5). Significant differences: *compared to control group (P < 0.05).

Fig. 4. Downregulation of Wnt signaling pathway by diabetes and chronic running can salvage these changes. A. Expression levels of Wnt ligands genes. Amounts of Wnt3, Wnt5a, and Wnt5b mRNAs in the plantaris were measured by qRT-PCR analysis. Target mRNA expressions were normalized to that of GAPDH and then plotted as the expression ratio relative to control group. B. Expression profiles of stabilized β-catenin. The protein expression levels of β-catenin were detected by Western blot. The right images represent the typical blot patterns of β-catenin and GAPDH. All values are expressed as the mean ± SEM (n=5). Significant differences: *compared to control group (P < 0.05), #compared to DB group (P < 0.05).
protein consistent with our data [42]. Therefore, we speculate that diabetes-induced reduction of Wnt signaling leads to decreased expression of MyoD occurring independently of Notch activity, and resulting in the inhibition of satellite cell activation. However, further analysis will be required to prove the hypothesis that Wnt signaling regulates MyoD transcription in advance of Notch activation in satellite cells.

Physical exercise has various physiological effects, including a reduction in body mass, increased maximum oxygen uptake, and metabolic improvements as well as an increased number of satellite cells in the skeletal muscle [43–45], although the effects as a countermeasure for DM-induced satellite cell dysfunction has not been previously reported. We confirmed that the increase in satellite cell and activation occurs in diabetic as well as normal mice. Previous studies showed that chronic running converts satellite cells from a quiescent to activated state via upregulation of the Wnt signaling pathway [23,35]. It has been reported that exercise-stimulated Wnt signaling directly modulates the chromatin structures of Myf5 and MyoD promoters, resulting in an acceleration of these gene transcriptions [23]. In the present study, we demonstrated that chronic treadmill running activates Wnt signaling in diabetic mice, suggesting that chronic running facilitates the conversion of satellite cells from a quiescent to activated state through the upregulation of Wnt signaling and chromatin remodelling of Myf5 and MyoD promoters in diabetic as well as normal mice. Conversely, the number of satellite cells are increased by chronic running in diabetic mice, although the activity of Notch signaling, which positively regulates the proliferation of satellite cells, did not change. One previous study reported that exercise increases the expression of Notch signaling components [46]. One possible explanation for this contradiction is that exercise-induced Notch activation is a transient response that returns to the baseline 18 h later. Another study suggested that the levels of HeyL and Dll4 were downregulated following 4 weeks of wheel running [23]. Therefore, exercise-stimulated characteristic changes in satellite cells are probably regulated by Notch signaling, although the detailed mechanism remains unclear.

Activated satellite cells immediately proliferate and then fuse to existing myofibers or generate new muscle fibers. However, we think that satellite cells did not fuse to muscle fibers, because there were no changes in the muscle weights and CSAs of muscle fibers following chronic running in the present study. According to our immunohistochemistry data, it is obvious that satellite cells were activated by exercise in both normal and diabetic mice. We suppose that chronic exercise in our model can improve the activation of satellite cells but not promote subsequent differentiation and fusion to muscle fibers. A previous study has shown that the increment of myonuclei following chronic downhill running, which induces muscle injury [17]. Another human study has reported that nonhypertrophic exercise increases the number of activated satellite cells but not myonuclei [47]. These findings suggest that exercise intensity is a key to promote satellite cell fusion to muscle fibers. To promote the subsequent myogenic program after MyoD activation, we need further investigations into exercise conditions, such as style, intensity, time, and term.

5. Conclusion

The current study revealed that diabetes mellitus gives rise to satellite cell dysfunction, including a decrease in the number of cell and an inhibition of satellite cell activation, and these effects were associated with the downregulation of Notch and Wnt signaling activities. In contrast, 4-week treadmill running increased the number and activation of satellite cells and salvaged diabetes-induced satellite cell dysfunction via upregulation of Wnt signaling. Our results suggest that physical activity may be effective in preventing the attenuation of the skeletal muscle by DM. However, further studies investigating the factors that link Notch and Wnt signaling pathways to changes in satellite cell properties by physical exercise are needed for a more complete understanding of the functions and intrinsic ability of satellite cells in adult myogenesis.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.004.

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