Biochemical and Biophysical Research Communications 463 (2015) 618–623

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of Wnt-10b on proliferation and differentiation of murine melanoma cells

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ARTICLE INFO

Article history:
Received 8 May 2015
Accepted 29 May 2015
Available online 5 June 2015

Keywords:
Melanoma
Wnt signal
Wnt-10b
Differentiation

ABSTRACT

In spite of the strong expression of Wnt-10b in melanomas, its role in melanoma cells has not been elucidated. In the present study, the biological effects of Wnt-10b on murine B16F10 (B16) melanoma cells were investigated using conditioned medium from Wnt-10b-producing COS cells (Wnt-CM). After 2 days of culture in the presence of Wnt-CM, proliferation of B16 melanoma cells was inhibited, whereas tyrosinase activity was increased. An *in vitro* wound healing assay demonstrated that migration of melanoma cells to the wound area was inhibited with the addition of Wnt-CM. Furthermore, evaluation of cellular senescence revealed prominent induction of SA-β-gal-positive senescent cells in cultures with Wnt-CM. Finally, the growth of B16 melanoma cell aggregates in collagen 3D-gel cultures was markedly suppressed in the presence of Wnt-CM. These results suggest that Wnt-10b represses tumor cell properties, such as proliferation and migration of B16 melanoma cells, driving them toward a more differentiated state along a melanocyte lineage.

1. Introduction

Melanoma, a type of skin cancer, has been shown to have an increasing rate of incidence and affected patients have a poor prognosis. The Wnt/β-catenin signaling pathway is known to be involved in melanoma formation and tumor progression, and Wnt expression has been demonstrated in melanoma cells [1–4]. Blockade of the Wnt-2 ligand was reported to induce apoptosis in melanoma cells [5], suggesting that canonical Wnt-2 signaling is a survival factor. On the other hand, it was also reported that melanoma cells expressing Wnt-3a, another canonical Wnt, showed suppressed proliferation *in vitro* and formed smaller tumors *in vivo* when implanted into mice [6], indicating that canonical Wnt-3a signaling evokes biological actions against melanoma cells. Thus, the functions of canonical Wnt signaling in melanoma remain controversial.

Wnt-10b, a canonical Wnt protein, was also reported to be strongly expressed in melanomas [4]. However, there are no reports documenting the roles of Wnt-10b in melanoma cells *in vitro*. In the present study, we investigated the biological effects of Wnt-10b on murine B16F10 (B16) melanoma cells using conditioned medium from Wnt-10b-producing COS cells (Wnt-CM). Our results showed that Wnt-10b decreased the proliferation and migration activity of B16 melanoma cells, and increased their tyrosinase activity. In addition, Wnt-10b induced senescence in B16 melanoma cells in monolayer cultures and inhibited the growth of cell aggregates in collagen 3D-gel cultures. Our findings suggest that Wnt-10b represses the tumor cell properties of B16 melanoma cells and drives them toward a more differentiated state along a melanocyte lineage.

2. Materials and methods

2.1. Preparation of Wnt-10b

Wnt-10b was prepared from a Wnt-10b-secreting COS cell line, which was established by introducing the cDNA gene, as previously described [7]. Briefly, Wnt-10b-expressing COS (Wnt-COS) cells were seeded into 10-cm dishes, then cultured in DMEM culture medium (Wako, Osaka, Japan). After 48 h, supernatant samples collected from those cultures were used as conditioned medium

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http://dx.doi.org/10.1016/j.bbrc.2015.05.110
0006-291X/

containing recombinant Wnt-10b (abbreviated as Wnt-CM) after filtration through a 0.22-μm filter membrane. Supernatants collected from cultures of parental COS cells were also used as Wnt-10b-free conditioned medium (abbreviated as COS-CM).

2.2. Cell culture

Murine B16F10 (B16) melanoma cells were purchased from ATCC. They were maintained in an atmosphere of 5% CO_2 and 95% humidified air at 37 °C in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Cell proliferation analysis

B16 melanoma cells were suspended in culture medium, then plated at a density of 2.5×10^3 or 1.0×10^4 cells per well in 24-well plates, and cultured with or without Wnt-10b. After 2 days, cell numbers were determined using the trypan blue exclusion method.

2.4. DOPA staining

B16 melanoma cells were plated at a density of 2.5×10^3 or 1.0×10^4 cells per well in 24-well plates, then cultured with or without Wnt-10b. After 2 days, cells were fixed with 4% PFA for 30 min at room temperature. After permeabilization in 0.1% Triton X-100 in PBS for 2 min, the cells were washed with PBS and incubated in 0.1% L-DOPA for 4 h at 37 °C. Finally, they were rinsed and their images captured using a microscope.

2.5. Scratch assay

Cells were grown in 6-well-plates until confluence. Cell migration was determined using an *in vitro* wound healing assay, as previously described [8]. The difference between widths measured at 0 and 24 h was determined using photos obtained with a phase-contrast microscope, with the migration of Wnt-10b-untreated cells expressed as 100%. The percentage of the filled wounded areas was calculated using the following formula: (a-b)/b*100, where “a” is the width at the beginning and “b” the width after 24 h. Values are shown as the mean of at least 3 independent experiments.

2.6. SA-β-gal staining

Cells were plated at a density of 2.5×10^3 in 24-well plates, then cultured with or without Wnt-10b. After 2 days, SA-β-gal staining was performed with a β-gal staining kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed in PBS and fixed for 10 min at room temperature with 0.5 ml of fixative solution, then washed and incubated overnight at 37 °C with a staining solution mix. Blue staining was observed under a microscope and the absolute numbers of β-gal-positive cells in 200-cell samples were determined.

2.7. 3D culture

B16 melanoma cells were incubated in 3D-suspension or collagen 3D-gel cultures with Wnt-CM or COS-CM for 2 days in 96-well spheroid plates (SUMILON, Tokyo, Japan) at a cell density of 2.5×10^3 per well. Collagen 3D-gel was prepared with a CellMatrix Collagen type I-A kit (Nitta gelatin, Osaka, Japan), according to the manufacturer's instructions. After 2 days, cells were fixed in 4% PFA for 30 min at room temperature and rinsed, then images were obtained using a microscope.

2.8. TOPFLASH assay

Involvement of the canonical Wnt signaling pathway was examined using reporter assays. Two reporter plasmids, pTOP-FLASH and pFOPFLASH, were kindly supplied by Dr. B. Vogelstein [9]. Cells were transfected with the reporter plasmids, then 4 h later the cell culture medium was removed, and replaced with medium with or without Wnt-10b. After 48 h of incubation, the cells were lysed and luciferase activity was quantified using a luciferase reporter assay kit (Clontech, Worcester, MA), as recommended by the manufacturer, and normalized using the level of β-gal as the internal control.

2.9. Transplantation

Male 8-week-old BALB/c nude mice were purchased from Japan SLC (Hamamatsu, Japan). Cells were re-suspended in PBS and 1 million cells in 50-μl suspensions were subcutaneously injected into the mice. After 1 week, recombinant Wnt-10b or PBS (50 μl) was subcutaneously injected at a site near the formed tumor every 2 days, then the tumors were harvested after 2 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nara Medical University.

2.10. Statistical analysis

Data obtained *in vitro* are expressed as the mean ± SD of 5 independent experiments. Statistical significance was examined using Student's *t* test.

3. Results

3.1. Proliferation of B16 melanoma cells attenuated by Wnt-10b

Mouse B16 melanoma cells were plated at a density of 2.5×10^3 or 1.0×10^4 cells per well in 24-well plates, and cultured in the presence or absence of Wnt-10b for 2 days (Fig. 1). While low-dose Wnt-10b (Wnt-CM 0.5x) did not affect the number of cells, high-dose Wnt-10b (Wnt-CM 1x) suppressed the proliferation of B16 cells in both cultures with different initial cell counts. When we compared between the cultures with Wnt-CM, cell proliferation was significantly suppressed in those with Wnt-CM 1x as compared to those with Wnt-CM 0.5x.

3.2. Tyrosinase activity increased by Wnt-10b

Cultured cells were fixed, permeabilized, and incubated in L-DOPA solution to examine their tyrosinase activity *in situ*. In cultures initiated with 1×10^4 cells per well, images captured by phase contrast microscopy showed a higher percentage of tyrosinase activity-positive cells in cultures with Wnt-CM 1x (about 40%) as compared to those in cultures with COS-CM 1x (about 20%) (Fig. 2A and B). In cultures initiated with 2.5×10^3 cells per well, up to 70% of the cells were estimated to have tyrosinase activity with Wnt-CM, whereas that was seen in fewer than 10% with COS-CM (Fig. 2C and D). Prominent induction of tyrosinase activity by Wnt-10b was observed in cultures with a low number of cells.

3.3. Wnt-10b inhibits migration activity of B16 melanoma cells

Cell migration was examined using an *in vitro* wound healing assay (Fig. 3). In cultures with COS-CM, the cells migrated to nearly the entire scratched area and covered the artificial wound surface after 24 h (Fig. 3A). However, cell migration into the scratched area was less complete in cultures with Wnt-CM as compared to those

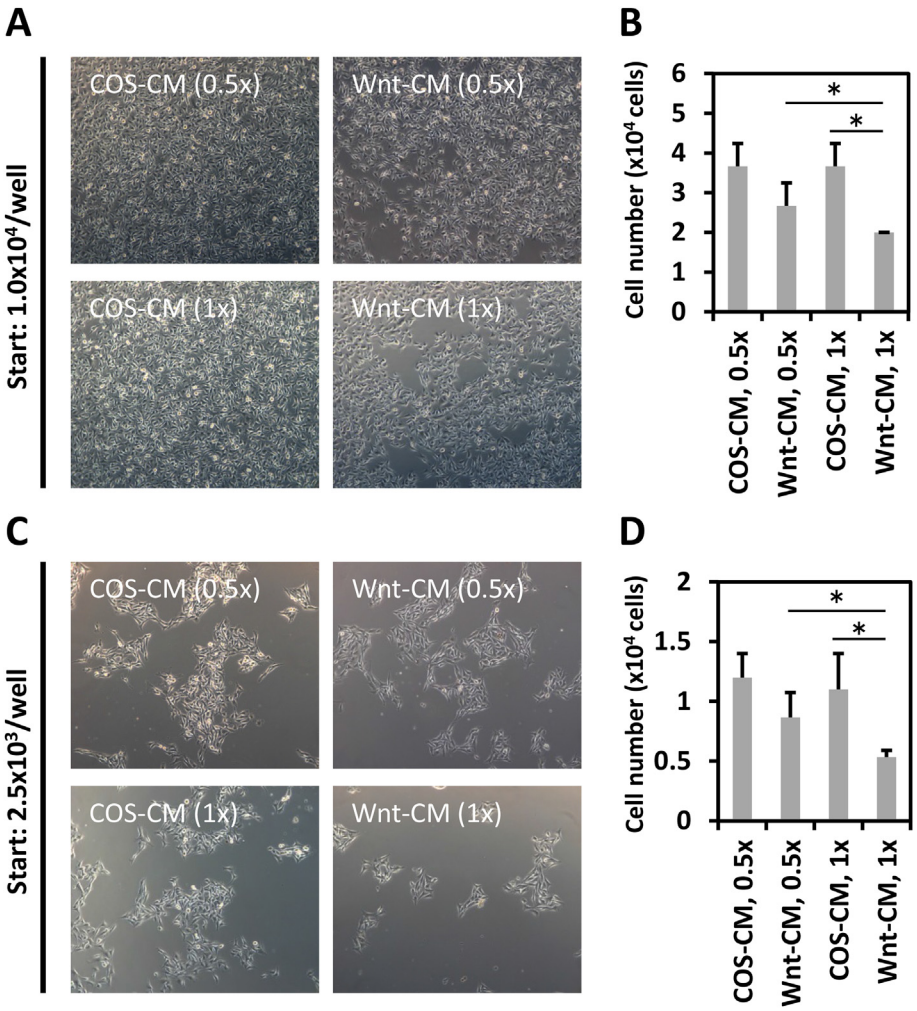

Fig. 1. Attenuated proliferation of B16 melanoma cells by Wnt-10b. Mouse B16 melanoma cells were cultured in Wnt-CM or COS-CM for 2 days at a density of 1.0×10^4 (A, B) or 2.5×10^3 (C, D) cells per well in 24-well plates. Microscopic images of representative 2-day cultures in (A) Wnt-CM and (C) COS-CM. (B, D) Cell numbers after 2-day cultures.

with COS-CM. Migration activity was attenuated by Wnt-10b (Fig. 3B).

3.4. Cellular senescence induced by Wnt-10b

We also examined whether cellular senescence was involved in attenuation of proliferation by Wnt-10b (Fig. 4). A greater percentage of cells was found to be SA-β-gal-positive in cultures with Wnt-CM as compared to those with COS-CM (Fig. 4A). Furthermore, the percentage of SA-β-gal-positive cells was markedly higher in cultures with Wnt-CM x1 as compared to cultures with Wnt-CM x0.5 (Fig. 4B).

3.5. Cell aggregate growth inhibited in collagen 3D-gel cultures

B16 melanoma cells were incubated in 3D-suspension or collagen 3D-gel cultures with Wnt-CM or COS-CM for 2 days (Supplementary Figure S1). In the simple 3D-suspension cultures, there was no discernable difference in regard to the growth of cellular aggregates between cultures with Wnt-CM and those with COS-CM. However, there was a marked difference seen in the collagen 3D-gel cultures, as aggregate growth in those was prominently inhibited by Wnt-CM.

4. Discussion

Wnt-10b has been shown to play various roles and have a wide range of biological actions, such as adipogenesis, bone formation, axis determination, and hair follicle (HF) formation, in skin tissue [10–13]. We previously reported that Wnt-10b promoted differentiation of cultured skin epithelial cells and also maintained the ability of dermal papilla cells to support trichogenesis [7,14,15]. Furthermore, it was recently shown that Wnt-10b promoted melanocyte maturation and pigmentation in hair bulbs of mouse HFs [16]. In contrast to the defined actions of Wnt-10b toward skin epithelial cells, dermal papilla cells, and melanocytes in the hair bulbs of HFs, the effects of Wnt-10b on melanoma cells are not well known, though its strong expression has been reported in melanomas [4].

In the present study, we investigated the effects of Wnt-10b on murine B16 melanoma cells using conditioned medium from Wnt-10b-producing COS cells (Wnt-CM) and found that Wnt-10b suppressed proliferation, upregulated tyrosinase activity, and inhibited the migration activity of B16 melanoma cells. These phenotypic changes strongly suggest that activation of Wnt/β-catenin signaling alters B16 cells by lessening their tumor cell characteristics and increasing those of differentiated melanocytes, though we did not examine gene expression related to pigment cell fate.

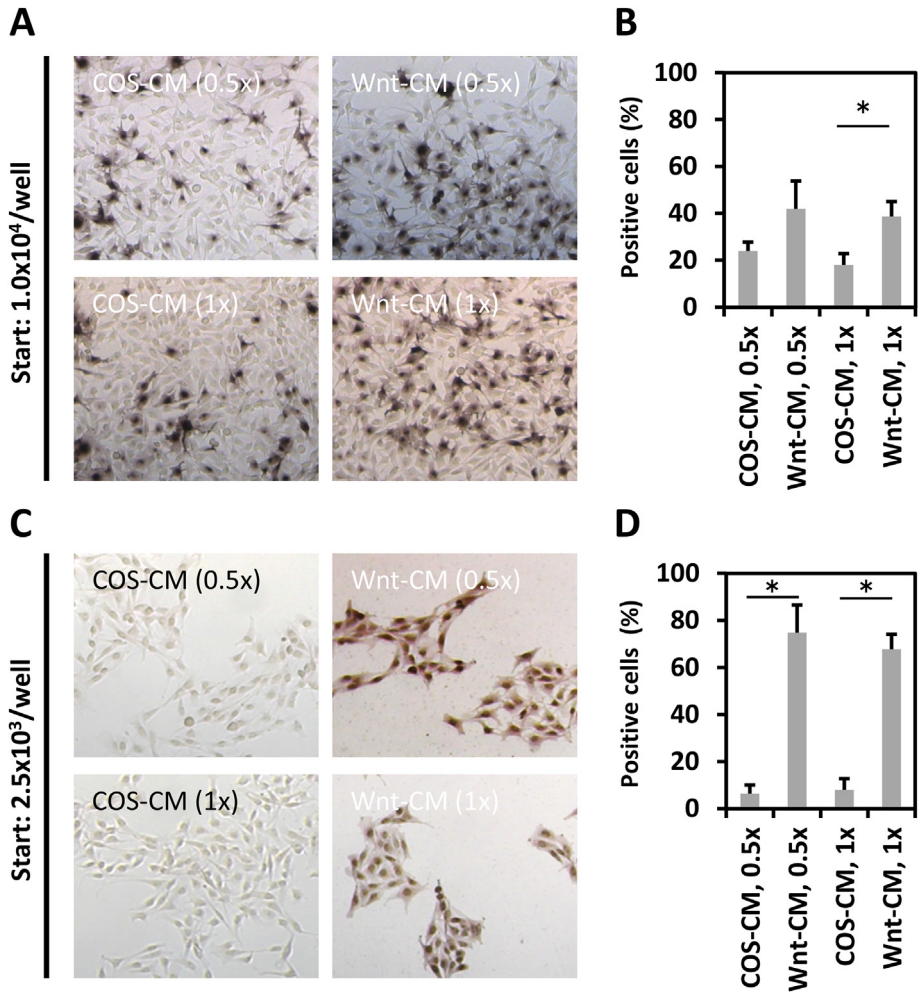

Fig. 2. Increased tyrosinase activity by Wnt-10b. Mouse B16 melanoma cells were cultured in Wnt-CM or COS-CM for 2 days at a density of 1.0×10^4 (A, B) or 2.5×10^3 (C, D) cells per well in 24-well plates, then the cells were fixed, permeabilized, and incubated in L-DOPA solution to detect tyrosinase activity. Microscopic images of representative 2-day cultures in (A) Wnt-CM and (C) COS-CM. (B, D) Percentages of tyrosinase activity-positive cells in 2-day cultures.

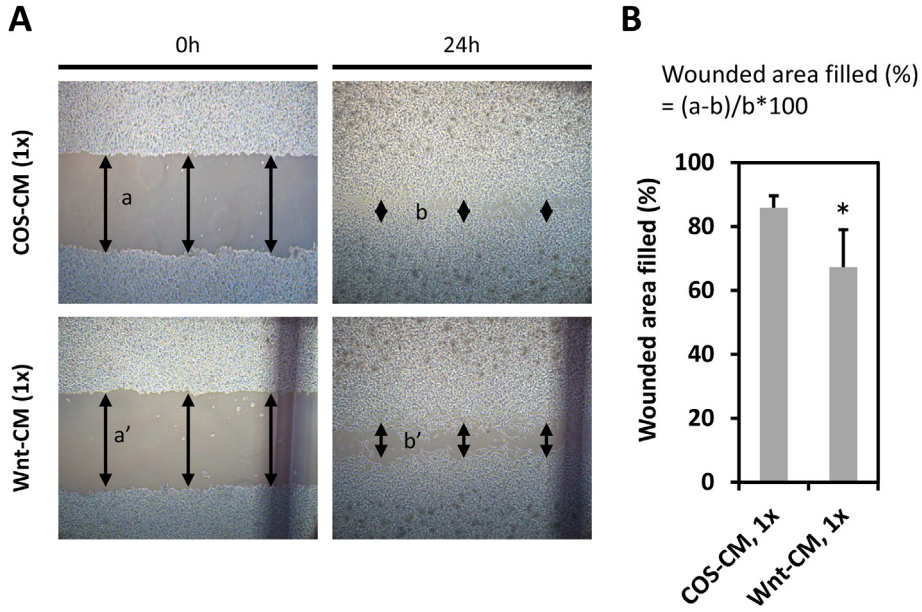

Fig. 3. Migration activity inhibited by Wnt-10b. Cell migration activity was examined using an *in vitro* wound healing assay. (A) Microscopic images of representative cultures at 0 and 24 h. (B) Wound area filled with migrated cells (%).

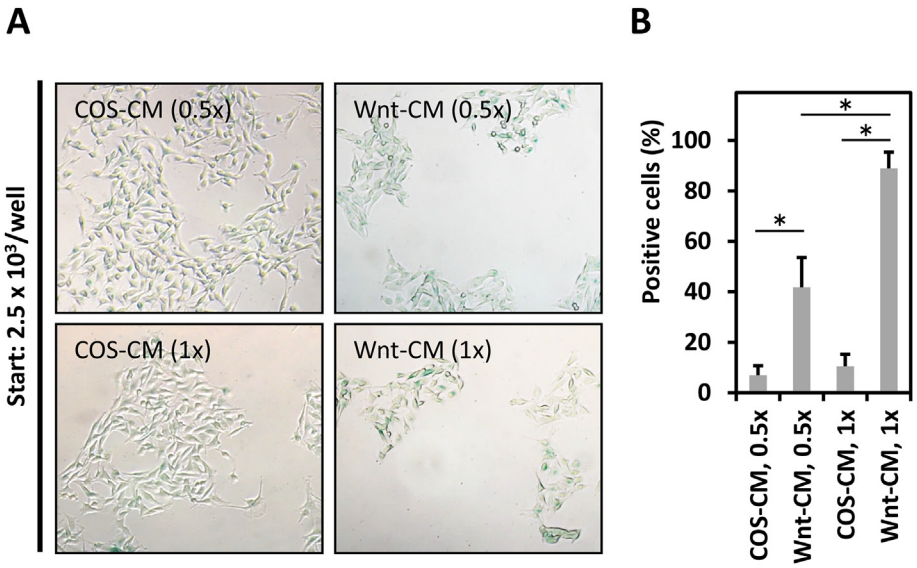

Fig. 4. Cellular senescence induced by Wnt-10b. Mouse B16 melanoma cells were cultured in Wnt-CM or COS-CM for 2 days at a density of 2.5×10^3 cells per well in 24-well plates, then SA-β-gal staining was performed. (A) Microscopic images of representative cultures after SA-β-gal staining. (B) Percentages of SA-β-gal-positive cells.

Recently, it was reported that Wnt/β-catenin activation was associated with decreased proliferation, while it promoted differentiation of melanoma cells expressing Wnt-3a, as shown by increased expressions of *Mitf*, *Trpm1*, *Met*, *Sox9*, and *Kit* [6]. Wnt/β-catenin signaling enhanced by 6-bromoindirubin-3'-oxime (BIO) was also shown to reduce proliferation and migration of melanoma cells [17,18]. In the present study, TOPFLASH assay results confirmed that treatment with Wnt-CM induced Wnt/β-catenin signaling in B16 melanoma cells (Supplementary Figure S2). Therefore, our results obtained with Wnt-10b-treated B16 melanoma cells are similar to those obtained with melanoma cells expressing Wnt-3a and BIO-treated melanoma cells.

Combined treatment of Wnt-3a and TNF receptor death-inducing ligand (TRAIL) was reported to enhance apoptosis as compared to treatment with TRAIL alone [19]. Thus, Wnt-10b may enhance apoptosis of melanoma cells in combination with TRAIL. However, there are no reports regarding apoptosis induction in melanoma cells by Wnts alone, while BIO has been shown to have no potentiating effects on TRAIL-induced apoptosis. Accordingly, we decided to examine senescence instead of apoptosis in B16 melanoma cells in order to elucidate the suppressed proliferation caused by Wnt-10b and found a remarkable induction of senescence in Wnt-10b-treated B16 melanoma cells (Fig. 4).

Although most previous *in vitro* studies were conducted with 2D cultures, it is known that cells behave differently when cultured in a 3D extra-cellular matrix. Therefore, we examined the effects of Wnt-10b on melanoma cells using collagen 3D-gel cultures. The growth of cell aggregates was prominently inhibited by Wnt-10b in collagen 3D-gel cultures, but not in 3D-suspension cultures, suggesting that growth suppression of melanoma cells by Wnt-10b is dependent on the extra-cellular matrix.

Finally, we conducted a preliminary experiment by transplanting melanoma cells into BALB/c nude mice to examine the effect of Wnt-10b on tumor growth (Supplementary Figure S3, Table S1). Tumor growth was inhibited by repeated administrations of Wnt-10b into subcutaneous tissue in the area of the mass. Taken together, our results suggest that Wnt-10b represses melanoma progression by inducing senescence and promoting differentiation via the Wnt/β-catenin signaling pathway.

In conclusion, we investigated the effects of Wnt-10b on murine melanoma cells and found that it suppressed their proliferation and migration activity, and upregulated tyrosinase activity. In addition, Wnt-10b was found to induce senescence in melanoma cells. Although the mechanisms of the effects of Wnt-10b on melanoma cells require additional analysis, we concluded that Wnt-10b plays important roles for inhibition of melanoma formation and tumor progression.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.110.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.110.

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