

Comparative Study of Anti-Oncogenic MicroRNA-145 in Canine and Human Malignant Melanoma

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ABSTRACT. MicroRNA-145 (miRNA-145; miR-145) is aberrantly expressed in most of human cancers and plays a significant role in carcinogenesis and cancer progression. In the current study, we focused on how miR-145 plays a role in canine and human malignant melanomas. MiR-145 was significantly downregulated in canine malignant melanoma tissues and canine melanoma cell lines, as well as human melanoma cell lines tested. The ectopic expression of miR-145 showed a significant growth inhibition in both canine and human melanoma cells tested, and the effect was achieved partly by suppressing c-MYC in canine melanoma LMeC and in human melanoma A2058 and Mewo cells. At the same time, a suppressive tendency on cell migration in canine melanoma KMeC cells and significant suppression of cell migration in human melanoma A2058 cells by suppressing FASCIN1 were also found. These findings suggest that miR-145 acts as a tumor suppressor in both canine and human malignant melanomas.

KEY WORDS: anti-oncogene, c-MYC, FASCIN1, malignant melanoma, microRNA-145.

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In the last decade, many studies have focused on the role of microRNA (miRNA; miR) in human cancer [5]. MiRNAs are a new class of short (approximately 21–25 nucleotides) noncoding RNAs that are involved in negative regulation of gene expression through sequence-specific base pairing with target mRNAs, usually in their 3'-UTR. Some miRNAs have been shown to promote cell proliferation and survival (acting as oncogenes), while others diminish cell proliferation and survival (acting as tumor suppressors). Dysregulation of the miRNAs in cancer may be useful for diagnosis, prediction of prognosis and therapy. Based on recent studies, the high-throughput methodologies used for study of miRNAs in human cancers can also be applied to dogs [4, 7, 13], and their reports indicate that the dog may prove to be an ideal model for the study of oncomirs because naturally occurring cancers in pet dogs and humans share many features, including histological appearance, tumor genetics, molecular targets, biological behavior and response to conventional therapies.

MiR-145 has been frequently reported as downregulated in various human cancers, including prostate cancer [15], bladder cancer [10] and colon cancer [3], as well as B-cell malignancies [2]. The ectopic expression of miR-145 in such cells exhibits a growth inhibitory effect [1, 18]. In human malignant melanoma (HMM), it is suggested that a

higher expression level of miR-145 is associated with longer survival after recurrence [19]. However, the role of miR-145 is still unclear in malignant melanoma.

It is important that the mechanisms of carcinogenesis in both CMM and HMM are clarified based on comparative oncology to evaluate whether CMMs can be a model as HMMs for development of a new therapeutic modality. In this study, we verified and discussed the roles of miR-145 in CMM and HMM.

MATERIALS AND METHODS

Dogs and tissue preparation: Twenty-three primary oral CMM tissues were obtained from dogs who had undergone biopsy for histological diagnosis of malignant melanoma at the Animal Hospital, Gifu University, between 2009 and 2010. Eleven normal oral mucosa tissues (4 tissues from 4 dogs with malignant melanoma and 7 tissues from 7 healthy adult dogs) were obtained as control tissues. These tissues were used for total RNA extraction. The characteristics of all the dogs are shown in Table 1. Harvesting of normal oral mucosa tissues was approved by the university's animal care and use review committee.

Cell culture and cell viability: CMM cell lines KMeC, LMeC, CMeC-1 and CMeC-2 were grown according to the procedure described previously [11]. CMM cell line MCM-N1 was purchased from DS Pharma Biomedical Co., Ltd (Suita, Osaka, Japan), and HMM cell lines A2058 and Mewo were obtained from Health Science Research Resources Bank (Osaka, Japan), and they were maintained

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Table 1. Characteristics of CMM tissues and normal oral mucosa tissues

CMM tissues for miRNA expression analysis			Normal oral mucosa tissues for miRNA expression analysis		
Dog	Age (years)	Breed	Dog	Age (years)	Breed
T1	15.4	Mongrel	N1 (T1)	15.4	Mongrel
T2	12.4	Mongrel	N2 (T2)	12.4	Mongrel
T3	5	Mongrel	N3 (T3)	5	Mongrel
T4	3	Labrador Retriever	N4 (T11)	9	Bernese Mountain Dog
T5	11.8	Beagle	N5	5.25	Beagle
T6	14.3	Labrador Retriever	N6	5.25	Beagle
T7	14	Yorkshire terrier	N7	5.25	Beagle
T8	12.7	Golden Retriever	N8	8.25	Beagle
T9	11.9	Mongrel	N9	3.5	Beagle
T10	15.8	Beagle	N10	3.5	Beagle
T11	9	Bernese Mountain Dog	N11	3.5	Beagle
T12	10.1	Beagle			
T13	11.9	Mongrel			
T14	13.6	Mongrel			
T15	9.1	Labrador Retriever			
T16	12.7	Miniture Dachshund			
T17	15.4	Shiba			
T18	9.3	Miniture Dachshund			
T19	12.6	Golden Retriever			
T20	11.1	Labrador Retriever			
T21	13	Miniture Dachshund			
T22	12.5	Labrador Retriever			
T23	12	Labrador Retriever			

according to the manufacturer's protocol. The number of viable cells was determined by performing the trypan blue dye exclusion test.

Normal human primary epidermal melanocytes (HEM), which were purchased from ScienCell Research Laboratories (Carlsbad, CA, U.S.A.), were cultured according to the manufacturer's protocol.

Transfection of melanoma cells with miRNA: CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells were seeded in six-well plates at a concentration of 0.5×10^5 cells per well the day before transfection. The mature type of miR-145 (Applied Biosystems, Foster City, CA, U.S.A.) was used for transfection of the cells, which was achieved by using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's Lipofection protocol. The nonspecific control miRNA (HSS, Hokkaido, Japan) sequence was 5'-GUAG-GAGUAGUGAAAGGCC-3', which had been used as a control for nonspecific effects in our past studies [1, 14]. The mature type of miR-145 used in this study was 5'-GUC-CAGUUUCCCCAGGAAUCCCUU-3'. The effects manifested by the introduction of miR-145 into the cells were assessed for 72 (LMeC), 96 (KMeC and A2058) or 144 (Mewo) hr after transfection.

Quantitative RT-PCR using real-time PCR: Total RNA was isolated from the tissues and cells by the phenol/guanidinium thiocyanate method with DNase treatment. After extraction, formaldehyde gel electrophoresis for total RNA was performed to examine the quality of RNA samples. To determine the expression of miRNAs, TaqMan MicroRNA Assays (hsa-miR-145, hsa-let-7a, and RNU6B; Applied

Biosystems) were used to reverse transcribe three mature miRNA sequences to cDNA. The miRNA sequences of miR-145 and *let-7a* in the dog were the same as those in the human (miRBase; <http://www.mirbase.org/index.shtml>). The RT reactions and real-time PCR were performed according to the protocol described previously [14]. Controls included reactions with no templates and no primers and the hsa-let-7a TaqMan assay, which is used as an internal control in study of the miRNA expression profile of canine chronic lymphocytic leukemia [7], and RNU6B TaqMan assays were used as the internal controls in this study.

To determine the expression levels of mRNA, total RNA was reverse transcribed with a PrimeScript[®] RT Reagent Kit (TaKaRa, Otsu, Japan). Quantitative PCR (qRT-PCR) was then performed with the primers specific for *c-myc* and *fscn1* by using SYBR[®] Premix Ex Taq[™] (TaKaRa). The primers for *c-myc* and *fscn1* were as follow: canine-*c-myc*-sense-558, 5'-CAG CGA GGA TAT CTG GAA GA-3'; canine-*c-myc*-antisense-830, 5'-CTC CAC ATG CAG TCC TGG AT-3'; human-*c-myc*-sense-1391, 5'-ATG CTG GAT CAC CTT CTG CT-3'; human-*c-myc*-antisense-1830, 5'-TCG TCG TTT CCG CAA CAA GT-3'; *fscn1*-sense-1111, 5'-AGC TGC TAC TTT GAC ATC G-3'; and *fscn1*-antisense-1359, 5'-CTG GAA GAC GTC ATA GCT G-3'. *GAPDH* was used as an internal control.

The relative expression levels of miRNAs or mRNAs were calculated by the $\Delta\Delta Ct$ method.

Western blotting: Total protein was extracted from tissues or whole cells by the procedure described previously [14]. Protein contents were measured with a DC Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.). Ten micrograms of

lysate protein for Western blotting of c-MYC or FSCN1 were separated by SDS-PAGE using polyacrylamide gels and electroblotted onto a PVDF membrane (PerkinElmer Life Sciences, Inc., Boston, MA, U.S.A.). The detailed method after blotting was described previously [14]. The antibodies used in this study were anti-human c-MYC rabbit monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.) or anti-human FSCN1 mouse monoclonal antibody (Abcam, Cambridge, UK) properly diluted with TBS-T containing 2% bovine serum albumin and 0.01% sodium azide. Loading control was done by re-incubating the same membrane with anti-human β -actin antibody (Sigma, St. Louis, MO, U.S.A.). The expression levels of FSCN1 were calculated by densitometry and expressed as the FSCN1/ β -actin ratio.

Transfection of CMM and HMM cells with short interfering RNA for *c-myc* or *fscn1*: CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells for *c-myc* knockdown and CMM (KMeC) and HMM (A2058) cells for *fscn1* knockdown were seeded into 6-well plates at a concentration of 0.5×10^5 cells/well on the day before transfection. Short interfering RNA (siRNA) for *c-myc* or *fscn1* (10 or 20 nM) was used for transfection of the cells. The sequences of the siRNA were UAG UCG AGG UCA UAG UUC CUG UUG G (siR-*myc*; Invitrogen) and UCU UCU UGG AGG UCA CAA ACU UGC C (siR-*fscn1*; Invitrogen). The effects manifested by the introduction of siRNA for *c-myc* (siR-*myc*) or *fscn1* (siR-*fscn1*) into the cells were assayed at 72 (LMeC), 96 (KMeC and A2058) or 144 (Mewo) hr after transfection. The knockdown effect of siR-*fscn1* was calculated by densitometry and expressed as the FSCN1/ β -actin ratio.

Cell migration inhibition assay: CMM (KMeC) and HMM (A2058 and Mewo) cells were seeded into six-well plates at a concentration of 1.0×10^5 cells/well and transfected with miR-145 or siR-*fscn1* (20 nM). After the cells grew to confluence, wounds were made by sterile pipette tips. Cells were washed with PBS and refreshed with serum-free medium to exclude the effect of cell growth facilitated by serum. After overnight incubation at 37°C, the cells were fixed with absolute methanol and stained by Wright-Giemsa stain.

Statistics: Each examination was performed in triplicate. The expression levels of miR-145 were compared between CMM samples and normal tissues and between HMM cells and HEM using the Student's *t*-test. Also, the expression levels of mRNAs in the cells transfected with miR-145 and those in the control and the data for cell migration inhibition assay were compared by using the Student's *t*-test. One-way ANOVA followed by the Tukey method was used for the cell count analysis. In all the statistics data, the level of significance was considered to be $P < 0.05$.

RESULTS

Downregulation and the cell viability suppressive effect of miR-145 in melanoma: First, we examined the expression

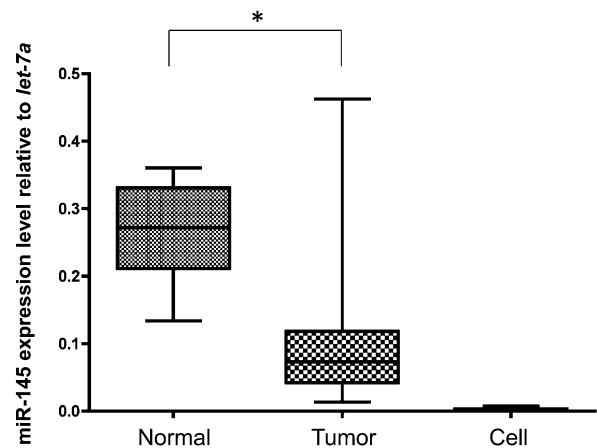


Fig. 1. Comparison of miR-145 expression level among normal canine oral mucosa tissues (normal), CMM tissues (tumor) and CMM cell lines (cell). The expression level of miR-145 was calculated by the $\Delta\Delta Ct$ method and normalized to *let-7a*. *: Significant difference between the bracketed samples (* $P < 0.001$).

levels of mature miR-145 in 23 CMM tissues, CMM (KMeC, LMeC, CMeC-1, CMeC-2 and MCM-N1) cells and 11 normal oral mucosa tissues by using TaqMan MicroRNA Assays. We chose both *let-7a* and RNU6B as internal controls. The expression levels of *let-7a* and RNU6B, which is generally used as an internal control, in CMM tissues and normal canine oral tissues were 21.16 ± 0.69 and 27.36 ± 0.92 (*Ct* values [mean \pm SD]). As shown in Fig. 1, in which *let-7a* was used as an internal control, the expression levels of miR-145 were significantly downregulated in CMM tissues (tumor) and CMM cells (cell) compared with those in normal canine oral mucosa tissues (normal) in the case of both *let-7a* and RNU6B as internal controls. The miR-145 expression levels of CMM cells were extremely reduced, and the average *Ct* values of the cells were as follows: 30.265 for MCM-N1, 34.93 for KMeC, > 37.0 for LMeC, 35.285 for CMeC-1 and > 37.0 for CMeC-2. So, we validated the effects of the ectopic expression of miR-145 on cell growth. The cell viability in CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells transfected with miR-145 was significantly decreased compared with that of the control (Fig. 2A). These findings altogether indicate that miR-145 negatively contributes to cell growth in the CMM and HMM cells tested.

It has already been reported by other groups that miR-145 targets *c-myc* and *fscn1* in human cancers [12, 18]. Therefore, we examined the expression profile of c-MYC and FSCN1 after transfection with miR-145. As shown in Fig. 3A, the expression levels of c-MYC protein after ectopic expression of miR-145 in the CMM and HMM cells tested were significantly reduced. Also, the expression levels of FSCN1 protein were markedly reduced except for HMM Mewo cells. On the other hand, *c-myc* expression levels were not significantly changed, and *fscn1* expression levels were significantly decreased (Fig. 3B). In order to clarify

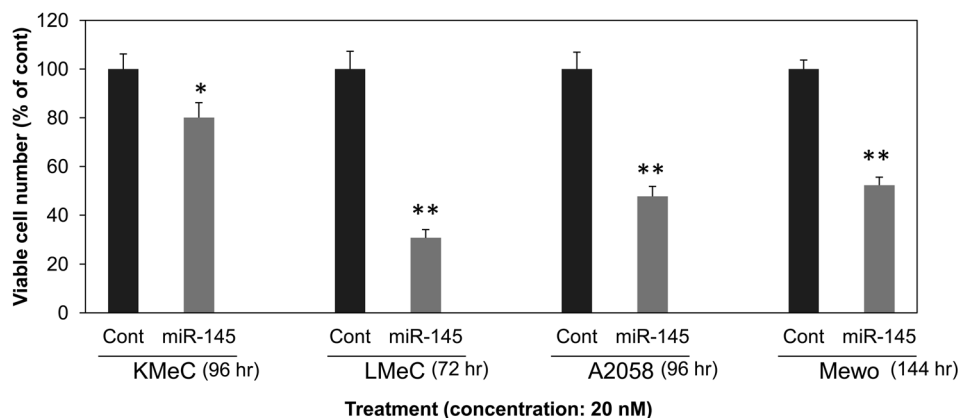


Fig. 2. The inhibitory effects on cell growth by ectopic expression of miR-145 in CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells. * and **: Significant difference compared with the control (* $P < 0.05$, ** $P < 0.01$). The viable cell number in the control was indicated as 100%.

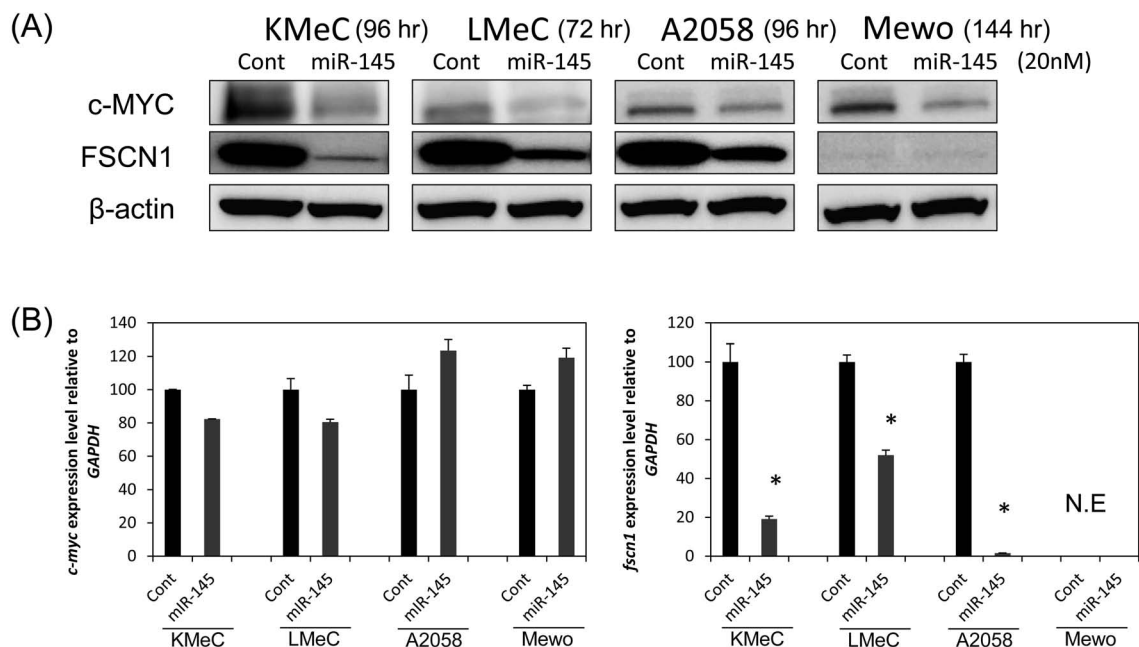


Fig. 3. (A) The expression levels of c-MYC and FSCN1, which are potential target genes of miR-145, in CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells transfected with control RNA or miR-145 (20 nM) by Western blot analysis. (B) The expression levels of *c-myc* and *fscn1* in CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells transfected with control RNA or miR-145 by qRT-PCR using a SYBR Green Assay. The expression levels of mRNAs were calculated by the $\Delta\Delta C_t$ method and normalized to *GAPDH*. *: significant difference compared with the control (* $P < 0.01$). N.E indicates no expression.

the function of miR-145 in HMM, we examined the expression levels of miR-145 in HMM cells. As well as CMM, the expression level of miR-145 in HMM cells was significantly downregulated compared with that in HEM cells (Fig. 4A). In order to examine the expression levels of possible target proteins in HMM cells, we tested the expression levels of c-MYC and FSCN1 by Western blot analysis. The expression levels of c-MYC were markedly higher in HMM cells than in HEM cells (Fig. 4B). On the other hand, the FSCN1

expression level of A2058 cells was slightly upregulated compared with that of HEM cells, and hardly any FSCN1 expression was found in Mewo cells (Fig. 4B).

c-MYC and FSCN1 contribute to cell viability and cell migration in melanoma cells: To validate whether c-MYC positively affected cell growth, we silenced it by transfection with siR-myc. The c-MYC expression level was clearly reduced by transfection, and the cell viability was significantly decreased in LMeC, A2058 and Mewo cells (Fig. 5)

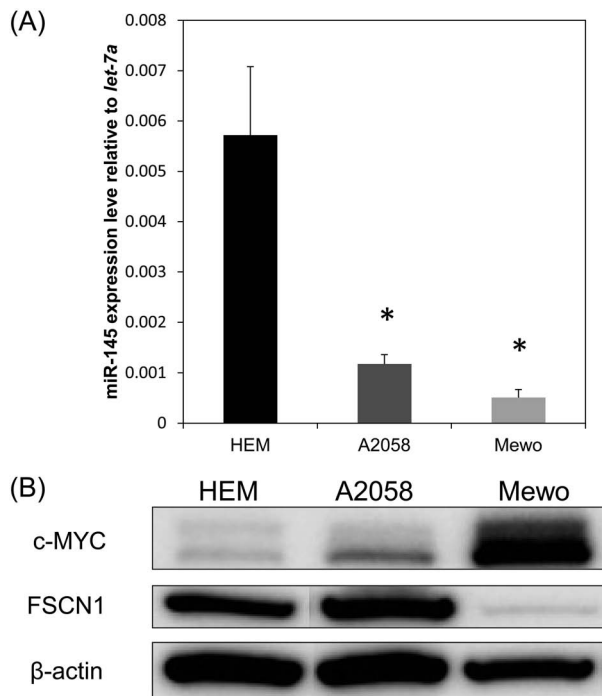


Fig. 4. (A) Comparison of miR-145 expression level between normal human epidermal melanocyte (HEM) and HMM (A2058 and Mewo) cells. The miR-145 expression level was calculated by the $\Delta\Delta Ct$ method and normalized to *let-7a*. *: Significant difference compared with the expression levels of HEM (* $P < 0.01$). (B) The expression levels of c-MYC and FSCN1 in HMM cells by Western blot analysis. c-MYC, which is a candidate target gene of miR-145, was markedly upregulated in HMM cells compared with the expression level in HEM cells.

but not in KMeC cells.

Next, to verify whether the ectopic expression of miR-145 inhibited cell migration through targeting of FSCN1, we performed a cell migration inhibition assay. As shown in Fig. 6A, the cell migration of KMeC cells transfected with miR-145 showed a tendency to be suppressed compared with that of the control ($P=0.10$). On the other hand, in the Mewo cells, which hardly expressed FSCN1 (Figs. 3A and 4B), cell migration was not disturbed. Additionally, in KMeC and A2058 cells with silencing of FSCN1, cell migration showed a tendency to be suppressed ($P=0.24$) in KMeC cells, and significant inhibition was observed in A2058 cells ($P=0.0003$) compared with the control, although cell viability was not significantly reduced (Fig. 6B).

DISCUSSION

In this study, miR-145 was downregulated in the CMM tissues and CMM and HMM cells tested. It was clearly indicated that miR-145 acts as a tumor suppressor by decreasing c-MYC expression in CMM (LMeC) and HMM cells and FSCN1 expression in CMM (KMeC and LMeC) and HMM (A2058) cells. We focused on *c-myc* and *fscn1* as target genes of miR-145. These genes have been validated as target genes of miR-145 in several kinds of human cancers based on a luciferase reporter assay by other groups [12, 18]. Consistent with past studies [12, 18], c-MYC expression was translationally suppressed in both CMM and HMM cells tested, and FSCN1 was suppressed through promotion of mRNA degradation in both CMM (KMeC and LMeC) and HMM (A2058) cells by the ectopic expression of miR-145. However, to validate *c-myc* and *fscn1* as target genes of miR-145 in MM and all kinds of canine cancers, a

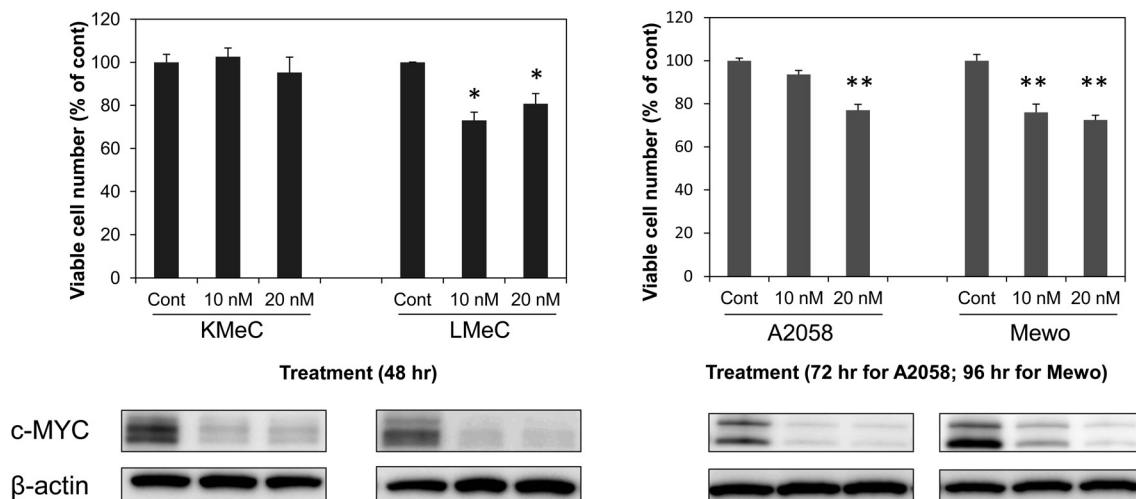


Fig. 5. The inhibitory effect on cell growth and the downregulation of c-MYC expression by *c-myc* knockdown in CMM (KMeC and LMeC) and HMM (A2058 and Mewo) cells. *: Significant difference compared with the control (* $P < 0.05$, ** $P < 0.01$). The viable cell number in the control was indicated as 100%.

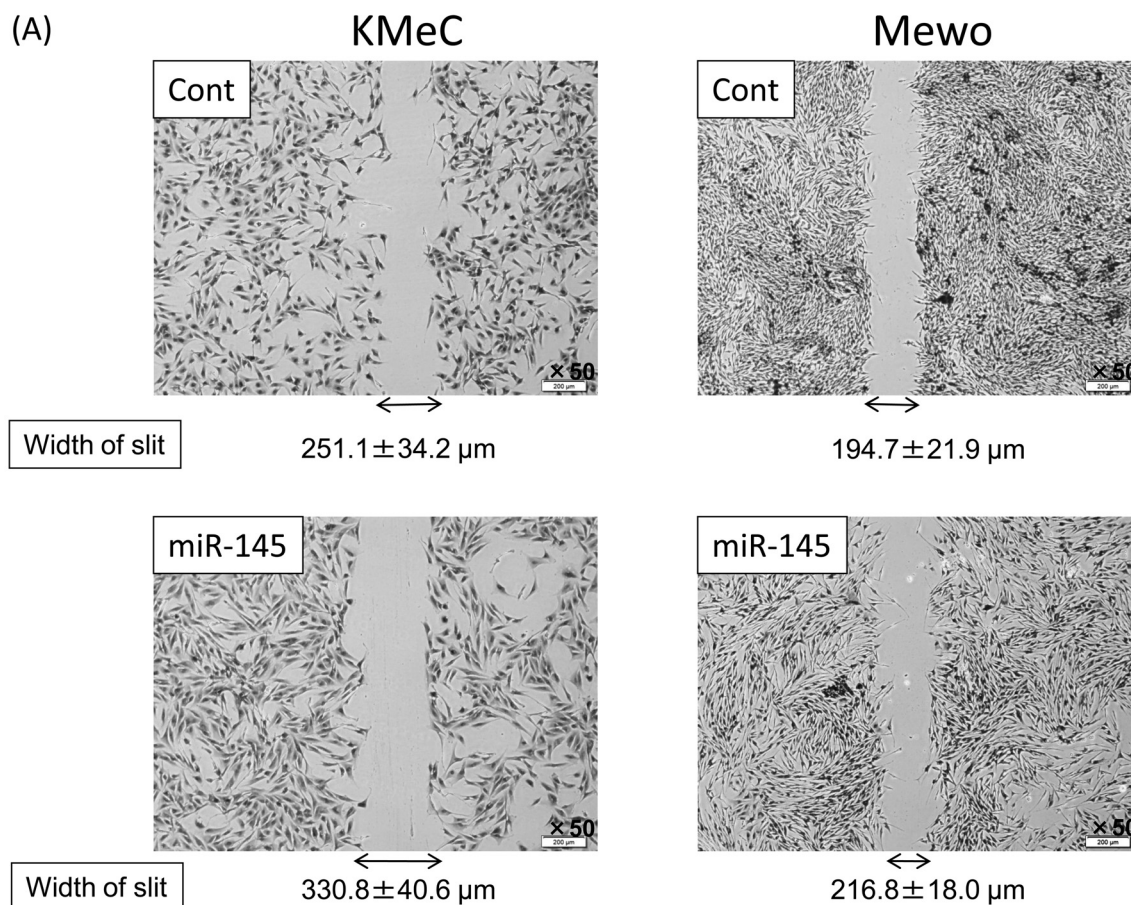


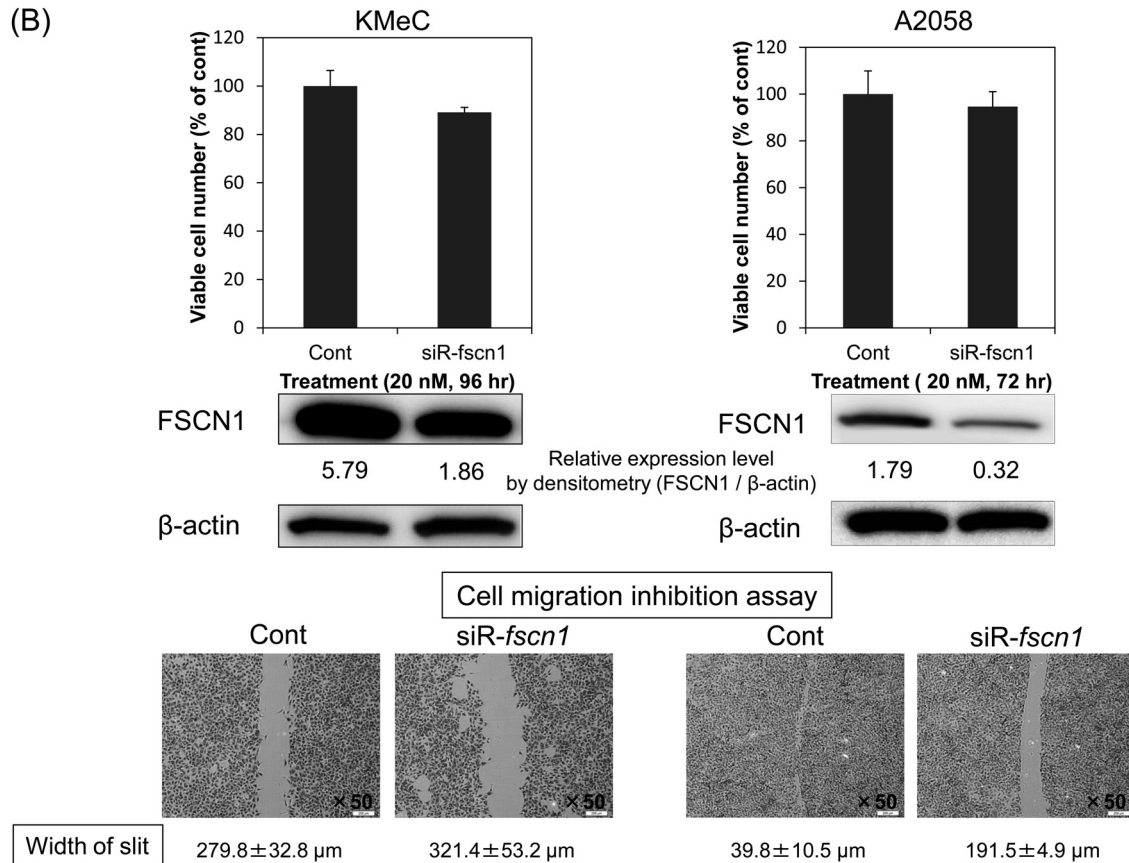
Fig. 6. (A) The inhibitory effect on cell migration by ectopic expression of miR-145. In a cell migration inhibition assay, the width of the slit in KMeC cells transfected with miR-145 tended to be narrower than that in the control ($P=0.10$). On the other hand, the width of the slit was unchanged between Mewo cells, which hardly expressed FSCN1, transfected with miR-145 and the control. (B) The effects on cell growth and cell migration by silencing of *fscn1*. The cell viability was almost unchanged. The viable cell number in the control was indicated as 100%. However, cell migration showed a tendency to be suppressed in KMeC cells ($P=0.24$) and significant inhibition was observed in A2058 cells ($P=0.0003$) transfected with siR-*fscn1*. The width of the slit was expressed as the mean \pm SD of 3 examinations.

luciferase activity assay is needed.

The protooncogene *c-myc* encodes a transcription factor that regulates ~10 to 15% of human genes and plays a central role in control of cell growth and apoptosis [6]. Deregulated expression of c-MYC has been detected in a wide variety of human cancers, including breast and colon, and is often associated with aggressive, poorly differentiated tumors [16]. It has been demonstrated that miR-145 is transactivated by p53 [18]. In that study, it was suggested that p53-mediated downregulation of c-MYC is, at least partially, due to the p53-mediated upregulation of miR-145. On the other hand, FSCN1 contributes to the organization of 2 major forms of actin-based structures: cortical cell protrusions that mediate cell interactions and migration and cytoplasmic microfilament bundles that contribute to cell architecture and intracellular movements. A recent burst of publications have indicated that overexpression of FSCN1

protein is observed in various kinds of human carcinomas. The upregulation of FSCN1 correlates with poor survival in non-small cell lung cancer and esophageal squamous cell carcinoma [8, 17].

In our preliminary data, both c-MYC and FSCN1 were also upregulated in CMM tissues compared with normal oral mucosa tissue. The findings shown in this study indicate that deregulation of c-MYC and/or FSCN1 contributes to carcinogenesis and/or progression in CMM and HMM. However, the mechanisms that involve other miR-145/target gene networks such as *ERK5* [9] or *OCT4* [20] may be associated with the carcinogenesis of CMM and HMM because suppression of c-MYC did not affect the cell growth in KMeC cells and FSCN1 expression was not observed in Mewo cells. In future study, validation of c-MYC and FSCN1 expression levels is needed in a larger sample set of CMM tissues.



In conclusion, the findings of the present study indicate that miR-145 can play a crucial role in carcinogenesis in both CMM and HMM. Clinical application of miR-145 as molecular targeting therapy for CMM and HMM may be useful in combination with surgery, chemotherapy or radiation therapy, although validation of effects *in vivo* is needed in the next step. Finally, we will certainly aim to control canine oral melanomas as the ultimate goal of this research.

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