Original Article

Involucrin, cytokeratin 10 and Ki67 expression in a three-dimensional cultured canine keratinocyte cell line in comparison to canine skin and cutaneous squamous cell carcinoma and a pilot study on custom-designed siRNA-INV transfection

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Abstract

Involucrin (INV) is a well-known early stage marker of terminal keratinocyte differentiation in humans. Besides its importance in human cancer, it has been predicted to be a prognostic marker in canine skin cancer. However, there is very little information available on canine INV. Therefore, for the first time, a three-dimensional (3D) cultured canine keratinocyte cell line was evaluated for the expression level of INV and two other cancer prognostic parameters, cytokeratin 10 (CK10) and Ki67, compared to that in canine normal skin (CN) and cutaneous squamous cell carcinoma (CSCC) cells. The INV protein and transcript expression levels were significantly ($p < 0.005$) higher in canine CN compared to in CSCC, whereas Ki67 expression levels were significantly ($p < 0.05$) lower in CN than in CSCC. The culture of a commercial canine keratinocyte cell line (CPEK; CELLnTEC Advanced Cell Systems, Switzerland) to produce the 3D epidermal skin was successfully achieved under an air-liquid interface for 14 d of culture. There was no statistical difference in the INV and CK10 expression levels between the 3D-cultured CPEK cells and CN. A custom designed INV siRNA transfected at 300 pmol showed the high efficacy of INV knockdown with minimum cytotoxicity to cultured CPEK cells, significantly reducing the INV mRNA expression level after 5 h transfection for up to 48 h. This is the first report on the custom design of siRNA transfection in monolayer cultured CPEK cells for INV gene knockdown in dogs and it could be useful for future studies on the carcinogenesis of CSCC.

Keywords: canine keratinocyte cell line, cutaneous squamous cell carcinoma, INV, CK10, Ki67

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Introduction

Skin cancer is a common cancer in not only humans but also in pets, especially canine and feline pets, as they share a similar environment and lifestyle to humans (Warland, 2011). Many spontaneously occurring cancers in pets have similar characteristics and behaviors to their human counterparts (Bongiovanni *et al*., 2009; Webb, 2009). The use of a companion animal is a suitable model to study human diseases, including cancers and cutaneous squamous cell carcinomas (CSCC), the main etiology of which is chronic ultraviolet light exposure (Dobson, 2011).

According to the records of the veterinary oncology clinic (The Small Animal Teaching Hospital; Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand), more than half of the dog and cat population in Bangkok stay outdoors. During 2010–2013, skin tumors comprised 31.5% of all the diagnosed tumor cases, and 22.5% of them were CSCC (82.9% in dogs, 17.1% in cats). After surgical treatment, almost all CSCC carrying dogs shared multiple reoccurrence within 2–3 months and in a more progressive state, because of the late initial detection (Assawawongkasem *et al*., 2016). The major risk factors for the occurrence of CSCC in dogs are a low pigmented skin and short coat hair, which are similar characters to white skinned or Caucasian people (Webb, 2009; Warland, 2011; Yan *et al*., 2011).

As is known, CSCC is induced by various cellular pathways, such as the loss of apoptosis, genetic instability and loss of cell cycle control resulting from changes in precursors and cellular signals of the cells (Melnikova and Ananthaswamy, 2005; Ratushny *et al*., 2012). Therefore, many precursors and signaling markers have been applied for the early detection and evaluation of cancer in bio-behavioral investigations and their cellular response to different treatments (Bongiovanni *et al*., 2009; Chanvorachote *et al*., 2012). Many useful markers have been reported recently, which are widely used for both human and canine SCC studies, such as MKI67 (Ki67) and cytokeratin 10 (CK10). The proto-oncogene Ki67 regulates cell proliferation and is a cell proliferation index marker whose expression is typically increased in tumors (Scholzen and Gerdes, 2000) and is related to rapid cell growth and recurrence of non-melanotic skin cancer (NMSC) (Nozoe *et al*., 2006; Martins *et al*., 2009) and poor prognosis in CSCCs (Assawawongkasem *et al*., 2016). The high molecular weight keratin, CK10, is normally expressed in the suprabasal layer of the epidermis or keratinizing stratified epithelia (Shibata *et al*., 2008) and in the differentiated areas of highly differentiated SCCs (Basta-Juzbasic *et al*., 2004), where other markers that identify cells of epidermal origin may be lost (Watanabe *et al*., 1995).

Involucrin (INV) is a cornified envelope precursor protein (Rice and Green, 1977; Eckert *et al*., 1997) that has previously been used as an early stage marker of human terminal keratinocyte differentiation (Zhang, 2017). Various studies have highlighted the use of INV expression as an indicator for wound re-epithelialization enhancement (Obedencio *et al*., 1999), a marker for atopic lesion with a reduction in cellular proliferation without apoptosis (Hertle *et al*., 1992; Jensen *et al*., 2004), a premalignant keratinocyte lesion marker (Caldwell *et al*., 1997; Li *et al*., 2000; Balasubramanian and Eckert, 2004; Nozoe *et al*., 2006), and an endogenous marker in hypoxia of SCC (Chou *et al*., 2004). This relationship of cell biological processes, such as keratinocyte differentiation, proliferation, apoptosis and transformation, in cancer progression, could be explained via the role of the Activator protein-1 transcription factor, an important regulatory protein in the epidermis (Eckert *et al*., 2004; Rorke *et al*., 2010). However, the use of INV for cancer prognosis in veterinary medicine is limited.

The keratinocyte culture model is a monolayer culture of co-cultured epithelial and mesenchymal cells that forms a three dimensional (3D) skin equivalent-organotypic culture. This *in vitro* skin model has been developed and used in experimental research, including pharmacological tests, the cosmetic industry, gene therapeutic approaches and as a cancer model in place of using animal models, such as use for local invasion and metastasis characterization of the biological behavior of cancers (Inoue *et al*., 2001; Kuhn *et al*., 2011).

It has previously been shown that air exposure is an important factor in the proliferation and differentiation of keratinocytes, which is why skin cultures are developed in vitro in an air-liquid interface method, especially for culturing CSCC cells (Inoue *et al*., 2001). A 3D culture has been established using canine primary keratinocytes. The CPEK canine keratinocyte cell line was established from normal trunk skin and retains its proliferative keratinocyte phenotype, including relevant gene expressions (Shibata *et al*., 2008; Yagihara *et al*., 2011).

Small interfering RNA (siRNA) is a class of short double-stranded RNA of 21–23 nucleotides containing 2−3 nt 3' overhangs, 5' phosphates, and free 3' hydroxyl-termini that guide the RNAi process resulting in a base-pairing-dependent down-regulation of the target gene expression by working together with other molecules to destroy the target mRNA after transcription (Storvold *et al*., 2006). The inhibitory RNA (RNAi) approach is a popular technique in genetic engineering research, both in cell culture and in living organisms. This technique is sometimes referred to as a "knockdown", to distinguish it from "knockout" procedures in which the expression of a gene is entirely eliminated (Meins, 2000; Macrae *et al*., 2006). The knockdown process of RNAi reduces specific target mRNA levels without damage to the gene structure (Alkharouf *et al*., 2007). Within oncology, previous studies have reported the efficacy of siRNA on P-glycoprotein inhibition, so as to increase the sensitivity to cancer chemotherapeutic agents in progressive cancer cells (Rumpold *et al*., 2005). In veterinary oncology, the efficacy of surviving gene silencing, which increases apoptosis in canine melanoma, was recently reported (Moriyama *et al*., 2010).

Therefore, the present study was designed to compare the expression of INV and other cancer prognostic markers (CK10 and Ki67) among canine normal skin (CN), CSCC and 3D-cultured CPEK cells. In addition, the pilot study on custom-designed

siRNA-INV transfection in cultured CPEK cells was also investigated.

Materials and Methods

Animal ethics: The canine CN, cut into 3 x 3 cm in pieces, was collected from the ventral skin of immediately deceased dogs, while CSCC masses were collected from the excisional CSCC masses following surgical operation. All procedures with animal sampling were conducted after approval from the Chulalongkorn University Animal Care and Use Committee (No.1431049) with the dog owner's consent, given as a signed consent form.

Quantitation of mRNA and protein expression pattern of INV, CK10 and Ki67 in CN and CSCC cells: Six samples, each of healthy dog CN and spontaneous CSCC, were obtained from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University.

The CN samples were used as the reference control and were first examined to confirm they were clinically normal and taken from dogs with no history of skin diseases, such as ectoparasitic infestation, skin allergies, bacteria or yeast infection and skin tumors. They were also confirmed to be free of papilloma virus by qRT-PCR (see section 2.3).

The CSCC carrying dogs were diagnosed by cytology and histology. All the collected samples of CN and CSCC were subsequently divided into three parts to be used for histopathology and INV protein expression by immunohistochemistry (IHC), immunofluorescence (IFC) on cryosections and mRNA expression by two-stage quantitative real time reverse transcriptase PCR (qRT-PCR).

Histopathology and INV protein expression by IHC: The CN and CSCC biopsied tissues (1 x 1 cm) were fixed in the 10% neutral buffered formalin (NBF) for routine histopathology according to standard laboratory procedures. Histopathological examination of CSCC was undertaken according to the classification system recommended by the WHO (Goldschmidt, 2002).

Serial sections of each specimen were subsequently prepared for INV protein expression by IHC as described previously (Theerawatanasirikul *et al.*, 2012b). The samples were serially incubated with mouse-monoclonal antibody (McAb) against human INV (Clone SY5, Abcam, UK) at a dilution of 1:1000, and then incubated by a polymer-based non-avidin-biotin system (EnVision™, Dako, Denmark) and visualized using 3, 3´-diaminobenzidine tetrahydrochloride (DAB) as the chromogenic substrate (Zymed Laboratories, UK). Positive and negative control slides were prepared using CN with and without primary antibody, respectively.

INV, CK10 and Ki67 protein expression by immunofluorescence (IFC): The freshly collected tissue samples (1 x 1 cm) were immersed in optimum cutting temperature compound, (Tissue-Tek OCT 4583 compound; Sakura, Tokyo, Japan) and rapidly frozen with dry ice. Then, 6-µm sections were cut using a routine cryostat microtome, Leica CM1800 cryostat (Leica, Heidelberg, Germany) at -20 °C. Each piece of cryostat tissue was placed on a slide and stained with methylene blue to observe the appropriate location and thickness.

The IFC was performed on 6-µm cryostat sectioned CN and CSCC on a coated slide, as per the protocol of the XL Biotech Co., Ltd. (Thailand). The sections were fixed in Fixing I solution (15 mins in dry ice), then in Fixing II solution (20 mins at 0 °C), and then incubated in 100 µL blocking buffer (FC blocking buffer, 4:100)/slide at 4 °C for 45 min. For the IFC staining, a ready-to-use anti-INV (ab68, SY5, Abcam) FITC-conjugated McAb was applied to the samples at 100 µL/slide for each primary Ab (one by one) of mouse McAb to CK10 (ab9026, DE-K10, Abcam), and rabbit polyclonal Ab (PcAb) to Ki67 (ab15580, Ki67, Abcam), IgG_1 and incubated at 4 °C for 60 mins (whereas mouse IgG_2 was used as an isotype control), and then washed three times with antibody binding buffer for 5 mins each. The tissue samples were then incubated with secondary goat PcAb against mouse IgG - H&L (ab7066, TR, Abcam) and rabbit IgG - H&L (ab6564, Cy5 ®, Abcam) for CK10 and Ki67, respectively as Table 1, at 4 °C for 60 mins, and then washed as above. Nuclei counterstaining was performed at the same time by the addition of DAPI. The immunolabeled sections were mounted with glycerol/phosphate buffered saline and observed by confocal laser scanning microscopy (CLSM) using a Leica DFC7000T (Germany) microscope at an excitation wavelength of 488 nm (for FITC) and 649 nm (for Cy5). Color images were acquired as optical sections and processed with LAS X imaging software.

Table 1 Primary and secondary antibodies used in Immunocytofluorescence techniques

Targeted protein	Primary antibody	Secondary antibody
INV	Anti-INV (ab68, SY5, Abcam) FITC-conjugated McAb	-
Ki67	Anti-rabbit polyclonal Ab (PcAb) to Ki67 (ab15580, Ki67, Abcam)	Anti-rabbit IgG1 - H&L (ab6564, Cy5 ®, Abcam)
CK10	Anti-mouse McAb to CK10 (ab9026, DE-K10, Abcam)	Anti-goat PcAb against mouse IgG1 - H&L (ab7066, TR, Abcam)

mRNA Quantitation of mRNA levels by two-stage qRT-PCR: The 1 x 1 cm pieces of the CN and CSCC samples in RNA later solution were extracted using RNAzol®RT (Sigma®), according to the manufacturer's instructions, and then subjected to DNase treatment using TURBO DNase-free (Ambion, USA). The concentration and purity of the obtained total RNA samples was determined using a Nano Drop ND-1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, USA) at an absorbance of 260 nm (A_{260}). Then

10 ng of total RNA was treated with DNase Free reagent (Ambion, Austin, TX) for 60 mins and reverse-transcribed to cDNA with SuperScript®III platinum® SYBR® Green one step qRT-PCR kit (Invitrogen, USA) at 37 °C for 120 mins using random primers. The second stage qRT-PCR used 5 µL of the obtained cDNA as a template and the canine CK10 (Involucrin) gene primer set (Theerawatanasirikul *et al*., 2012a), while papilloma virus samples were investigated using the MY09/MY11 (PP1) and FAP59/FAP64 (PP2) primer sets and amplified as described previously (Waropastrakul *et al*., 2012; Paolini *et al*., 2013). The human oral papilloma in formalin fixed paraffin embedded tissues (FFPE) were used as a control (kindly provided by Department of Pathobiology, Faculty of Science, Mahidol University). All primer information is given in Table 2.

Table 2 Primers used in the qRT-PCR in this study

Gene	Primers (5´-3´)	Product (bp)
INV[a]	F AAAGAAGAGCAGGTGCTGGA R TGCTCACTGGTGTTCTGGAG	203
CK10[a]	F TTGAGACGCACTGTTCAAGG R AGCTCGGATCTGTTGCAGTT	168
Ki67[b]	F CCCACCTGTCCTGAAGAAAA R TGTGGTCACTTCCAGTTGGTT	88
PP1[c]	F GCMCAGGGWCATAAYAATGG R CGTCCMARRGGAWACTGATC	452
PP2[c]	F TAACWGTIGGICAYCCWTATT R CCWATATCWVHCATITCICCATC	484

Primer sequences are from [a]Theerawatanasirikul *et al.* (2012a), [b](Vascellari *et al*., 2012) and [c]Waropastrakul *et al.* (2012).

The second stage qRT-PCR was performed using a Swift® spectrum 48 Real Time Thermal Cycler (Esco, Singapore) with Taqman reagents (Applied Biosystems, Foster City, CA). The expression level of the housekeeping gene ß-actin was used to normalize the expression of the genes of interest using the ΔΔCt method (Wong and Medrano, 2005). Standard curves were generated using the data from 10-fold serial dilutions of the total RNA to confirm the efficiency of the assay and evaluated from the R^2 value.

Culture and characterization of 3D cultured CPEK: The commercial canine keratinocyte cell line, CPEK, was maintained in complete CnT-09 medium (CELLnTEC Advanced Cell Systems, Switzerland). The 3D-culture was performed as previously reported (Yagihara *et al*., 2011). The CPEK cells were then thawed, harvested and the medium changed every 2 d without antibiotic/antimycotics at 37 °C in a 5% (v/v) CO_2 atmosphere. When the CPEK cells were 80% confluent (after 3 or 4 d culture) they were washed and transferred to a Millipore polycarbonate insert (0.4 µm pore size, 12 mm in diameter) at a seeding density of 5 x 10^5 cells/mL. Inserts were placed in a multi-well plate and immersed with CnT-PCT medium (3D-culture medium) until the level inside and outside the insert was the same and the cells were submerged in it to form an air-liquid interface. The cells were then cultured for 21 d, changing the medium every alternate day. Cultured cells were collected on days 14 and 21.

The growth rate of the monolayer cultured CPEK cells was observed daily under phase contrast microscopy. The cells were harvested for cell counting by a hemocytometer. Viability was evaluated by the trypan blue stain exclusion method or using the LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher, L3224) based upon calcein-AM and propidium iodide (PI) staining. For the 3D-culture, the cultured CPEK samples were fixed in 10% NBF and embedded in paraffin after 14 and 21 d in culture for routine histology.

The histological observation was processed with hematoxylin and eosin (H&E) staining to observe the growth of the 3D-culture under light microscopy. The IFC and mRNA quantitation analyses were conducted as previously described (sections 2.2 and 2.3).

Alteration of cultured CPEK with INV gene silencing

Custom designed siRNA:Double-stranded, siRNAs (21-mer) targeting INV were designed and synthesized using the web-based program of Bioneer's Custom siRNA synthesis service (https://us.bioneer.com /sirna/custom-sirna-ex.aspx) to target the untranslated region (UTR) of INV, where the top three candidate sequences from the INV siRNA design (INV-1, INV-2, and INV-3) were selected and synthesized (Table 3).

Titration and optimization of the siRNA transfection: For the siRNA transfection, monolayer cultured CPEK cells were transfected with the transfection reagent using a proprietary dosing strategy with a siRNA concentration of 50, 100, 150, 300, and 600 pm/10^6 cells for each of INV-1, INV-2, INV-3, and all three pooled siRNAs. The appropriate conditions of siRNA transfection and titration were investigated on day 3 of the monolayer cultured CPEK cells (at 40–60% cell confluency) as per the AccuTarget® Custom siRNA guideline (www.bioneer.com).

Transfection of siRNA was performed using lipofectamin™ RNAiMAX (Invitrogen®, USA) according to the manufacturer's protocol. Cells were transfected at approximately 40–60% cell confluency with either the selected INV and using cultured cells as a control in the same condition as the manufacturer's instructions. After 5 h of transfection, the transfection

mix was washed out, fresh medium was added and the cells were harvested 0, 6, 12, 24, and 48 h later, while their growth rate and characteristics were observed under phase contrast microscopy. The cells treated with siRNA and the control (non-transfected cells) groups were each further divided into two groups; one for RNA isolation (to evaluate changes in gene expression levels) and the other for determining cell viability using the LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher, L3224). Triplicate samples were conducted in each group.

Table 3 Target mRNA sequence of the three designed siRNAs for canine INV

Gene		Primers (5´-3´)
INV-1	SENSE	GAACAGCUGGAACAGGAAA(dTdT)
	ANTISENSE	UUUCCUGUUCCAGCUGUUC(dTdT)
INV-2	SENSE	GCUAGAGCAGAUAGGAGCA(dTdT)
	ANTISENSE	UGCCUCCUAUCUGCUCUAGC(dTdT)
INV-3	SENSE	AGCACCAGAAGCAGGAGGU(dTdT)
	ANTISENSE	ACCUCCUGCUUCUGGUGCU(dTdT)

After obtaining the appropriate (optimal) transfection parameters (concentration and time), these conditions were then selected to seed the transfected CPEK cells to form the 3D cultures. Briefly, the transfected medium was changed after 2 d, and when the cells were 80% confluent (after 3 to 4 d) they were washed and transferred to a Millipore PCF polycarbonate insert at a seeding density of 5 x 10^5 viable cells/mL. Inserts were placed in a multi-well plate and immersed with 3D cultured medium and cultured for 21 d, changing the medium every alternate day.

Statistical analysis: The relative quantification of gene transcripts, obtained from the qRT-PCR, was analyzed between groups by one-way analysis of variance (ANOVA) and Turkey-Kramer test using the SPSS version 15 for Windows (IBM Corporation, New York, USA). Mean, standard deviation (SD), and coefficient of variation of each marker were determined. Statistical significance was accepted at the $p < 0.05$ level.

Results

Protein expression pattern (IFC and IHC analyses) and mRNA quantitation (two-stage qRT-PCR) of INV, CK10, and Ki67 in CN and CSCC: The CN samples in this study were collected from six healthy dogs (one male, five females), while six spontaneous CSCC (three males, three females) dogs were used for the CSCC samples. The dogs ranged between 5–15 years old and there was no prevalence in sex or breed.

The IHC staining pattern of INV in CN demonstrated a strong granular like intra-cytoplasmic pattern in the whole layer of the nucleated epidermis in the upper spinous and granular layer of the stratified squamous epidermis (Figure 1a). The IFC staining pattern of CN, observed using the LAS X computer color merging program, for INV (Figure 1b, 1c) and CK10 (Figure 1b, 1d) revealed a positive intracytoplasmic staining pattern in the whole layer of the nucleated epidermis, while an intra-nuclear staining pattern of epidermal cells was found for Ki67 (Figure 1e).

Out of the six CSCC of dog patients, four, one, and one had well-, moderate-, and poorly-differentiated SCC, respectively. The histopathological appearance of CSCC consisted of large neoplastic keratinocytes. The nuclei were hyperchromatic and nucleoli varied in size and prominence. Formation of keratin pearls and intracytoplasmic keratin proteins was well identified. In the well-differentiated CSCC, proliferating neoplastic squamous epithelial cells were arranged in compact cords or nests of varying size, with abundant connective tissue and lamellate keratin pearls in the center of the islands. On the other hand, the poorly-differentiated CSCC consisted of highly proliferating cells that showed a high degree of anaplasticity with an absence of cell nests and keratin pearls or keratinized cells.

The IHC staining pattern of INV revealed a low staining brownish color at the keratin pearl area with DAB-hematoxylin counterstaining in the well- and poorly-differentiated CSCC cells (Figure 2a, b). The IFC staining pattern of the poorly-differentiated CSCC revealed that INV was weakly stained in some areas compared to in CN (Figure 2d), while CK10 showed a strongly positive intracytoplasmic staining pattern (Figure 2e) and Ki67 had a strong intra-nuclear staining pattern (Figure 2f).

The mRNA expression level of INV, CK10, and Ki67 in the CN and CSCC, as determined by the qRT-PCR analyses (Table 3), was in accord with the IFC staining pattern. Specifically, the INV mRNA expression level was higher in CN than in CSCC, while Ki67 mRNA expression was lower in CN than in CSCC. The CK10 mRNA expression level was higher in CSCC than in CN. All the CN and CSCC samples were negative for papilloma virus in the PCR assays using the PP1 and PP2 primers.

Culture and characterization of the 3D cultured CPEK: The monolayer CPEK cells, harvested using the Cnt-09 media, were successfully cultured to 80–90% confluency in 3–4 d and then subcultured to 80% confluency before being used to establish the air-liquid interface culture for 21 d. At day 14, the cells had a good proliferation rate and had undergone differentiation, where their morphology appeared like

epidermal skin (Figure 4a). After 21 d of culture, the cells ceased to grow and started to degenerate (Figure 4b). By IFC staining, the cells were revealed to be positive for INV and CK10 expression but negative for Ki67 (Figure 4c-e).

Expression level of INV, CK10, and Ki67 mRNA (qRT-PCR analysis): The mRNA levels determined by the two-stage qRT-PCR analysis complemented the IFC staining results, where there were no statistical differences in the INV and CK10 expression levels between the 3D cultured CPEK and CN, while Ki67 expression was not detected by either method (Figure 5).

Alteration of cultured CPEK with INV gene silencing: The treatment of the monolayer cultured CPEK with the INV-1 siRNA reduced the mRNA levels of INV more than that by the INV-2, INV-3, and pooled siRNA treatments (Figure 6). The results revealed that 300 pmol was the lowest possible concentration of INV-1 siRNA that was still highly effective at INV mRNA knockdown and showed a minimum cytotoxicity. For the exposure time, a gradual decrease in INV mRNA expression occurred from 0–48 h. At 48 h, the harvested cells showed a slightly increased INV expression level. In terms of cell viability, assayed using the LIVE/DEAD® Viability/Cytotoxicity Kit, the treatment with 600 pmol siRNA resulted in a higher proportion of dead cells (red color), at about 20%, than with the other concentrations where the transfected CPEK cells demonstrated a good cell viability (Figure 7). There was no significant difference in the Live/Dead cell assay at the different time exposures.

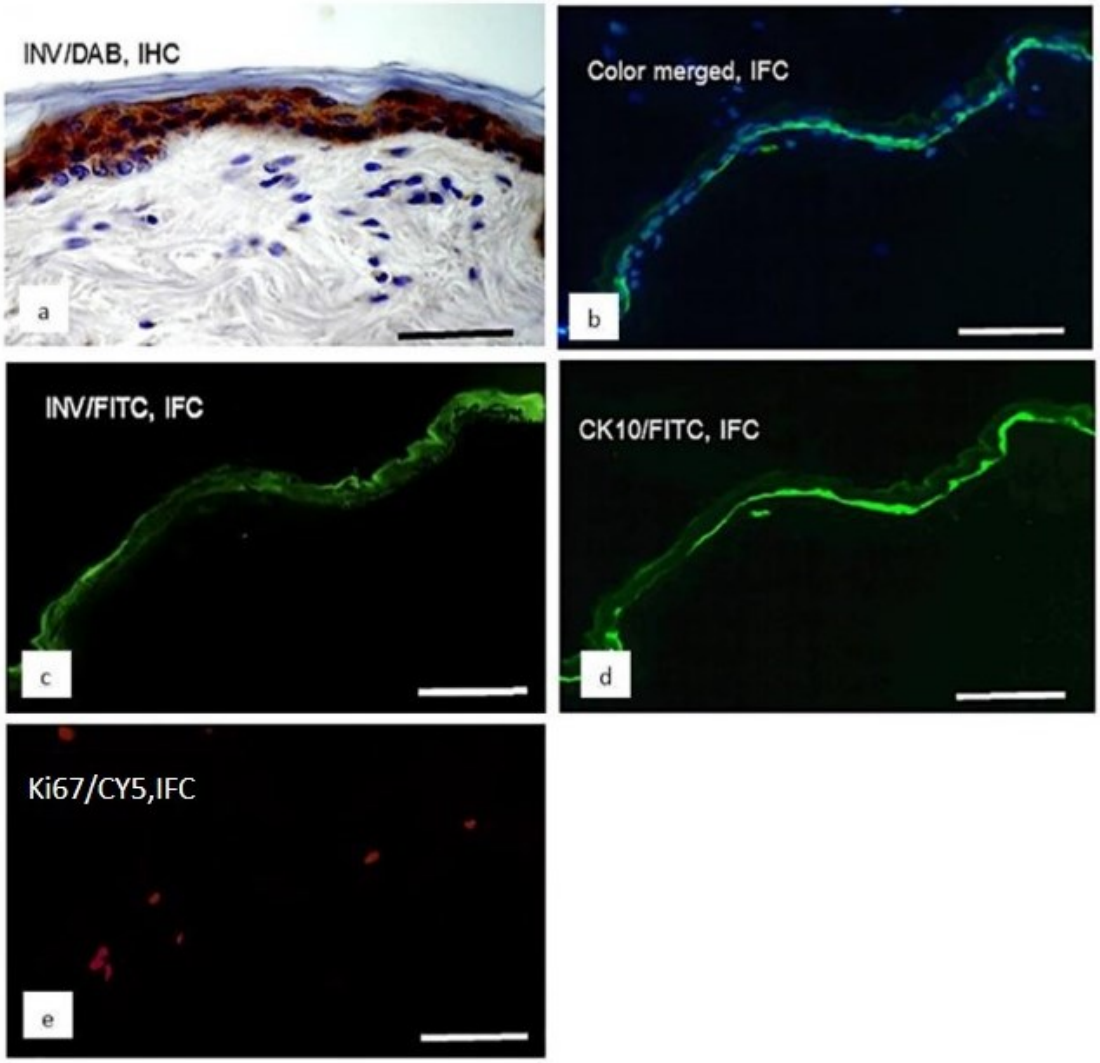

Figure 1 The IHC and IFC staining patterns in CN. (a) The INV staining pattern showed a strong granular-like intracytoplasmic pattern in the whole layer of the nucleated epidermis (IHC using DAB and hematoxylin counterstaining, Bar = 25 µm). (b) Color-merged IFC staining pattern of INV/CK10/Ki67/DAPI demonstrated their presence in the whole layer of nucleated epidermis in a similar pattern to that seen in the IHC analysis, and (c-e) IFC images of (c) INV/FITC, (d) CK10/FITC, and (e) Ki67/cy5. Color/Bar = 25 µm.

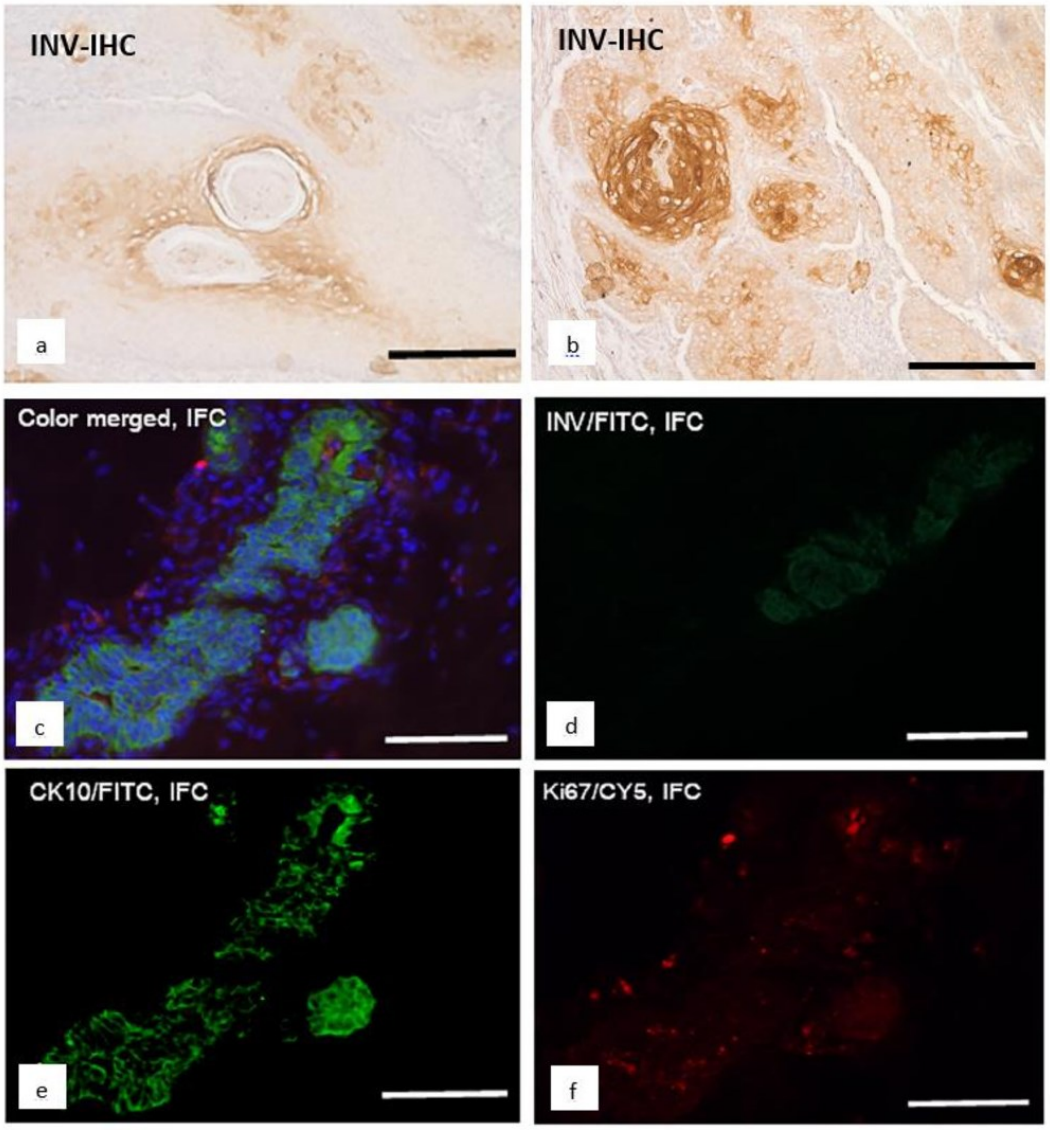

Figure 2 The IHC and IFC staining in spontaneous CSCC showing (a) an intensely positive INV intracytoplasmic staining in the neoplastic cells and in the keratin pearl in well-differentiated CSCC, and (b) weak to absence INV staining of neoplastic cells in poor-differentiated CSCC (DAB and hematoxylin counterstaining, Bar = 25 µm). (c-f) Color merged IFC showing (c) INV/CK10 /Ki67/DAPI (Color/Bar = 25 µm), (d) INV/FITC, (e) CK10/FITC, and (f) Ki67/cy5.

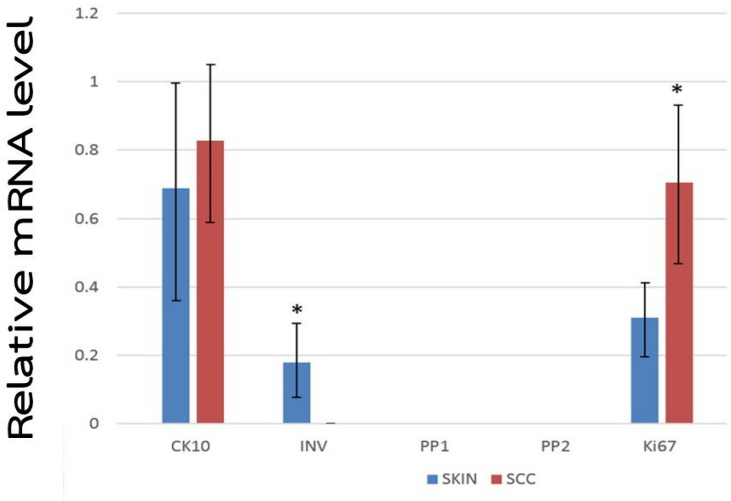

Figure 3 Histogram comparing the mRNA levels (qRT-PCR) of INV, CK10, Ki67 in canine CN and CSCC. The INV expression is higher in CN than in CSCC, while Ki67 expression is significantly lower in CN than in CSCC. All six samples were negative for papilloma virus (PP1 and PP2 primer). Data is shown as the mean ± SD, normalized to ß-actin as the housekeeping gene. * Statistically significant difference ($p < 0.05$).

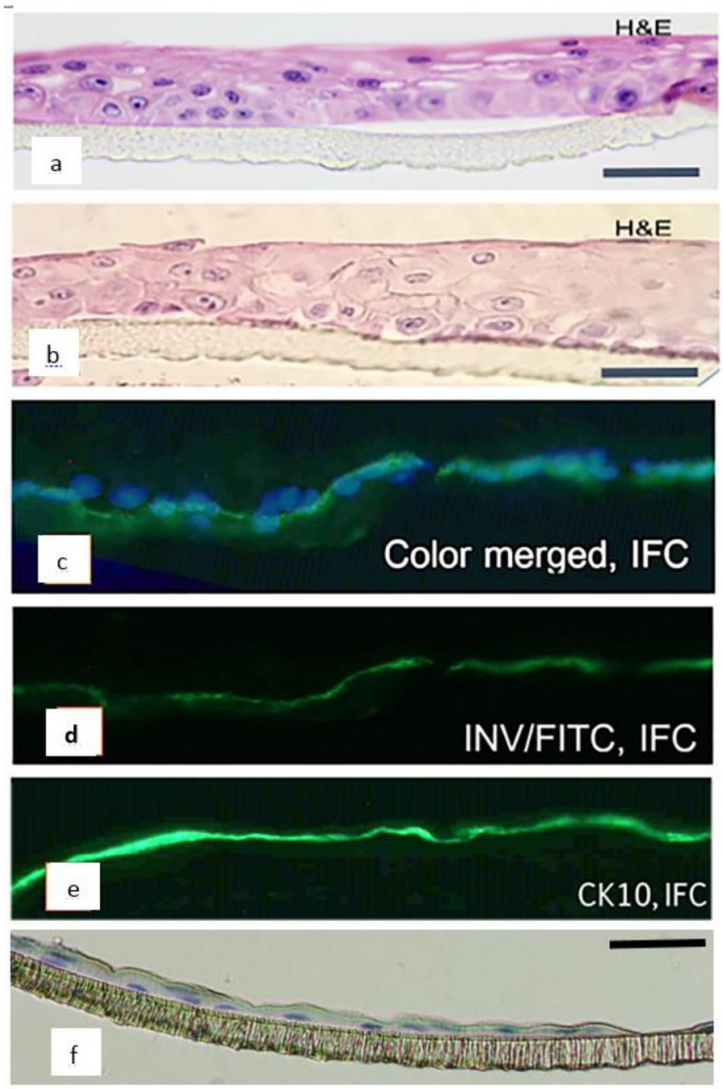

Figure 4 Histopathology of the 3D air-liquid interface cultured canine CPEK cell line, which became a stratified squamous epithelium with an epidermal-like structure on the Millipore polycarbonate on (a) day 14, while (b) by day 21 the CPEK became swollen and started to degenerate (H&E, Bar = 25 µm). (c-f) Representative IFC images (x 400) at day 14 of the of 3D air-liquid interface cultured CPEK showing the color merged (c) INV/CK10/Ki67/DAPI, (d) INV/FITC, and (e) CK10/FITC. (f) The CPEK could not develop as a 3D culture after siRNA-INV transfection, at day 14 (methylene blue, Bar = 25 µm).

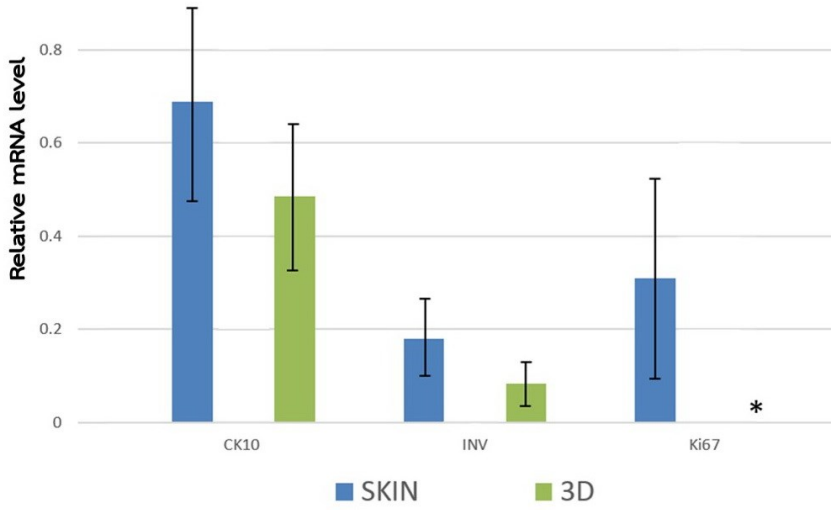

Figure 5 Expression levels of mRNA (qRT-PCR) of INV, CK10, and Ki67 in the canine CN and 3D-cultured CPEK. There was no statistical difference in INV and CK10 expression between the 3D-cultured CPEK and the CN. Negative results for Ki67 expression in the 3D-cultured CPEK was evident. Data is shown as the mean ± SD, normalized to ß-actin as the housekeeping gene.
* Statistically significant difference ($p < 0.05$).

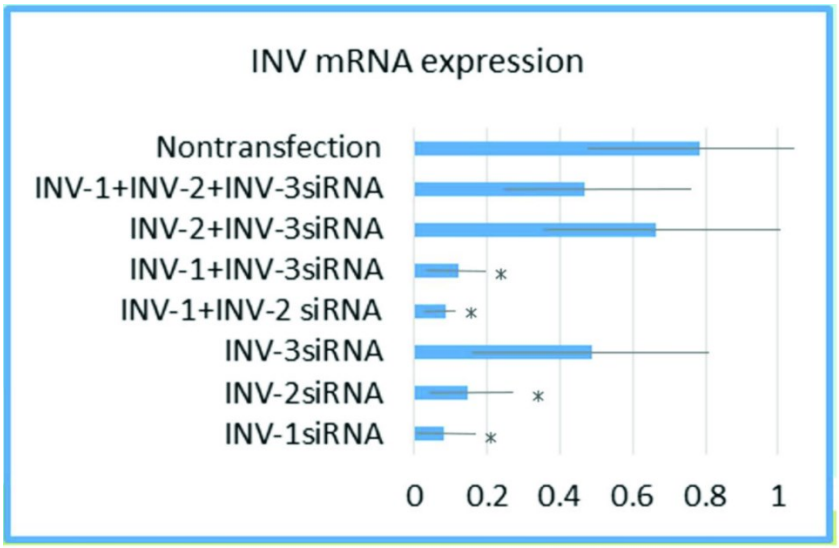

Figure 6 Demonstration of the silencing (knockdown) efficacy of the three custom designed siRNAs targeting the canine INV expression in monolayer-cultured CPEK. The INV mRNA levels were measured by qRT-PCR based on the delta Ct calculation. Data ic shown as the mean ± SD (three replicates), normalized to ß-actin as the housekeeping gene. The single siRNA of INV-1 showed the best knockdown of INV expression.

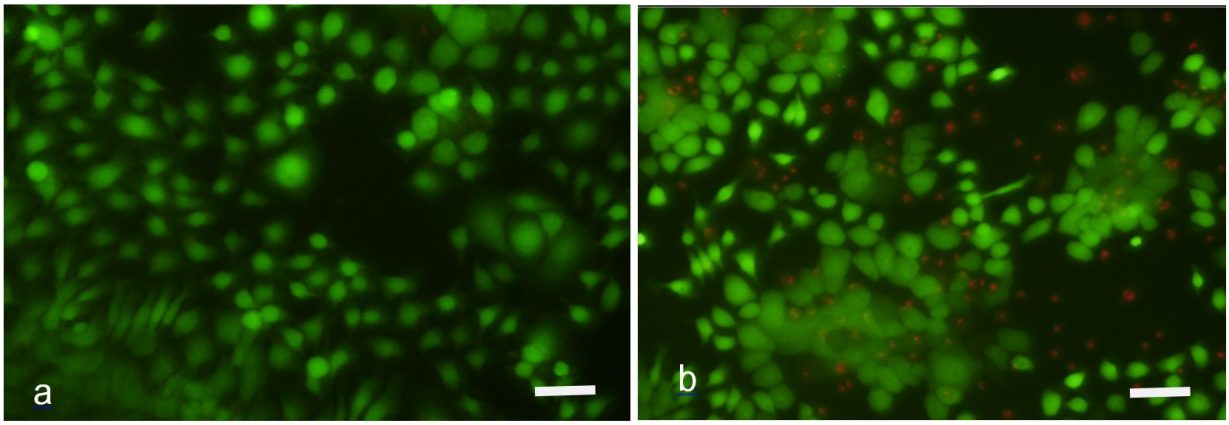

Figure 7 Calcein-AM and PI (LIVE/DEAD® Viability/Cytotoxicity Kit) based cell viability. Live cells fluoresce a bright green color, whereas dead cells with compromised membranes fluorescence in red-orange color. Representative IFC images of (a) 300 pmol and (b) 600 pmol INV-1 siRNA transfected cultured CPEK cells (Bar = 20 µm). Cell viability was approximately 90% and 80% at 300 and 600 pmol INV-1, respectively.

Discussion

The incidence of canine skin tumors in the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University (Bangkok, Thailand), was 31.5% of all the diagnosed tumor cases (Sailasuta, 2013). The CSCC samples in this study were collected from dogs of 9.5 ± 3.5 y of age, which is similar to a previous report where the age of affected dogs was 10.7 ± 3.2 y with no sex or breed prevalence (Assawawongkasem *et al.*, 2016). With respect to the breed of dog, they varied from case to case. It has recently been demonstrated that cornified envelopes (involucrin and filaggrin), keratins (keratin 10 and 5), and their mRNA expression levels were similar among various breeds and sexes in CN and so all breeds could be used as CN samples (Theerawatanasirikul *et al.*, 2012b).

Although the major risk factor of CSCC, similar to humans, is solar or UV radiation, papilloma virus is a minor risk and has been reported in 15% of CSCC dogs and chronic dermatosis, which are abnormal at molecular and genetic levels (Webb, 2009; Chandrashekaraiah *et al.*, 2011; Luff *et al.*, 2016). Therefore, all of the selected sample cases in this study (6 cases), both CN and CSCC, were screened for papilloma virus by qRT-PCR, as previously described (Waropastrakul *et al.*, 2012), and found to be negative. Thus, none of the the CSCC cases had a relationship to papilloma virus.

The staining pattern of INV in the FFPE biopsied CN and CSCC specimens was similar to that in previous studies (Theerawatanasirikul *et al.*, 2012b; Assawawongkasem *et al.*, 2016), where the INV expression level of our results was clearly decreased in all the CSCC groups, except for in the well-differentiated CSCCs group where it was increased.

The protein expression staining pattern corresponded with the mRNA levels, where the mRNA expression level of INV was higher in CN than in CSCC, while Ki67 was lower in CN than in CSCC. This is in accord with a previous study that

demonstrated by IHC staining INV expression in almost all cutaneous neoplasm in dogs decreased significantly ($p < 0.001$) compared to in CN (Assawawongkasem *et al.*, 2016). Moreover, in CSCC, there was an inverse correlation ($p < 0.0001$) between INV and the proliferative index, Ki67 level, and histology grading (Assawawongkasem *et al.*, 2016). As INV is a marker of terminal differentiation of the epidermis (Eckert *et al.*, 1997), this implied that defective terminal differentiation of the transformed stratified squamous epithelial cells could be recognized by the decreased INV expression level, although the mechanism(s) remains unknown. However, INV has been examined in human dermatology, including in normal epidermal renewal and various immune diseases and genetic defects of the skin (Hertle *et al.*, 1992; Jensen *et al.*, 2004). It has been established in a human tumor model that INV is an oxygen-regulated protein and a useful endogenous marker of hypoxia (Chou *et al.*, 2004), which is significantly correlated to the clinicopathological signs of SCC patients (Nozoe *et al.*, 2006).

Generally, CK is a marker of differentiated keratinocytes (Shibata *et al.*, 2008). The expression of CK in the epidermal neoplasm is usually conserved during a malignant transformation of the epidermis, whereas other markers of epidermal origin may be lost. Thus, the identification of CK in a poorly differentiated tumor can help to establish the epithelial tumor origin of its malignancy (Watanabe *et al.*, 1995). The high molecular weight keratin, CK10, is normally expressed in the suprabasal layer of the epidermis or keratinizing stratified epithelia and in differentiated areas of highly differentiated squamous cell carcinomas (Basta-Juzbašić *et al.*, 2004). Although CK10 expression was clearly demonstrated in the CN, its expression level was increased in the CSCC samples, which is opposite to that observed for INV expression.

In the CSCC samples, the protein and mRNA expression levels of INV showed a decreased expression, whereas Ki67 showed a significantly ($p < 0.05$) increased expression. The relationship between Ki67 and INV expression has been reported before in proliferative, pre-neoplastic and neoplastic skin in humans (Caldwell *et al.*, 1997). Additionally, it was found that a decreased expression of INV was related to the rapid growth and recurrence in NMSC cells in humans (Nozoe *et al.*, 2006; Martins *et al.*, 2009) and in canines SCCs (Assawawongkasem *et al.*, 2016).

The development of a 3D-cultured keratinocyte system in an air-liquid interface was performed in this study and found to promote cell proliferation and differentiation, as reported previously (Inoue *et al.*, 2001; Kuhn *et al.*, 2011). These previous studies showed that air exposure is an important factor for the proliferation and differentiation of keratinocytes and it is possible to use it as an experimental animal skin model (Inoue *et al.*, 2001). The cultured CPEK cells developed a monolayer in 2 d without antibiotic/antimycotic at 37 ^{o}C in 5% (v/v) CO_2 atmosphere, while the 3D-cultured CPEK cells revealed cell proliferation and differentiation characteristics at 14 d similar to a normal epidermis. However, at 21 d the cells had ceased to grow and started degenerating, which is in agreement with previous studies that (i) developed a canine epidermis equivalent by interfacing epidermal keratinocytes and fibroblasts embedded into a biomatrix collagen gel, where the skin equivalent was observed in vitro up to 3 weeks, with an optimal growth period of 2 weeks (Serra *et al.*, 2007; Yagihara *et al.*, 2011), and (ii) using 3D-cultured keratinocytes from a foot pad in a collagen gel to develop a skin equivalent in 5 d (Yamazoe *et al.*, 2007). Thus, the 3D-cultured CPEK under an air-liquid interface successfully developed an artificial canine epidermis in 14 d. This is shorter than a dog or human epidermis in vivo, which have a turnover rate of 22 d and approximately 28 d, respectively, (Yager, 1991).

In the 3D-cultured CPEK, the expression levels of INV, CK10, and Ki67 protein and mRNA were determined by IFC and qRT-PCR, respectively. The INV and CK10 revealed a positive expression in CSCC, while Ki67 was negative, which is similar to the CN. There were no statistical differences in the INV and CK10 expression levels between the 3D-cultured CPEK and CN, and so the 3D-cultured CPEK was quite similar to CN in both histology and gene expression.

In contrast, the negative results of Ki67 mRNA expression might be due to the characteristics of the 3D-cultured CPEK samples, which had ceased proliferation and proceeded to differentiate. In agreement with a previous report, Ki67 showed a weak positive staining at the bottom-most layer of CPEK cells cultured in a collagen gel (Yagihara *et al.*, 2011). This difference might be due to the different cell culture systems of an air-liquid interface and a feed layer, which facilitated the cellular adhesion and its differentiation (Nigro *et al.*, 1997).

The currently available commercial siRNA against INV are all designed for humans, rats and mice (https://www.scbt.com/scbt/product/involucrin-sirna-m-shrna-and-lentiviral-particle-gene-silencers) . Therefore, specific canine INV siRNA was designed using the online custom siRNA Design Tool User Guide of Bioneer pacific (https://us.bioneer.com /sirna/custom-sirna-ex.aspx). From the top three selected siRNAs (INV-1, INV-2, and INV-3), INV-1 at 300 pmol was found to be the lowest possible concentration that showed a high efficiency in INV knockdown with a low cytotoxicity in the monolayer-cultured CPEK cells, while a 5 h transfection exposure time resulted in a 70% decreased INV expression level during 0–48 h. After 48 h, the monolayer-cultured CPEK cells showed a slightly increased level of INV expression level; and so up to 48 h was the knockdown period in this experiment.

For future optimization, it is noted here that it was previously reported that a single siRNA transfection in HeLa cells gave a more than 80% knockdown that lasted 5–7 days post-transfection (Li, 2008). In addition, higher siRNA concentrations did not result in an increased knockdown level or duration, but were likely to cause off-target effects. Repeated siRNA transfection has, in some cases, been reported to improve the knockdown during selected time points post-transfection, depending on the designed siRNA and transfection method (Reynolds *et al.*, 2004; Fakhr *et al.*, 2016).

The custom designed canine INV siRNA could be further developed to improve the knockdown efficiency (%) and duration. Regardless, 300 pmol INV-1 siRNA was the lowest concentration in this study that was suitable for INV knockdown with a minimum cytotoxicity. The 3D-cultured CPEK did not develop when using the siRNA-INV transfected CPEK cells (Figure 4f and unpublished data), which might be due to the transfection process that destroyed the adhesion functions of CPEK or the loss of INV, which is an important cornified envelope precursor protein (Rice and Green, 1977; Eckert *et al.*, 1997). In addition, the expression of the adhesion protein, E-cadherin, was previously shown to be correlated with the INV expression (Silva *et al.*, 2017). Thus, it may be assumed that CPEK cannot develop to a 3D-culture in the absence of the INV protein.

In conclusion, the obtained results demonstrated that the protein and mRNA expression level of INV are different between CN and CSCC. The 3D-cultured CPEK in an air-liquid interface system was successfully developed as an artificial epidermis in dogs after 14 d of culture, with a similar histology and INV and CK10 protein and mRNA expression patterns to those in CN. This is a pilot study on the design of canine siRNA for INV gene knockdown and transfection in monolayer-cultured CPEK cells, which could be useful for further study on the carcinogenesis of CSCC.

Acknowledgements

The authors thank the staff of the Companion Animal Cancer Research Unit (CAC-RU), Faculty of Veterinary Science, Chulalongkorn University, and the Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand. Grant support was provided by the Chulalongkorn University graduate scholarship, the 90th Anniversary of the Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) academic year 2014, and in part by the Asia Research Center (ARC) fund, 2012.

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