Identification of markers of squamous cell carcinoma in sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2 heterozygote mice keratinocytes with altered Ca^{2+} signaling

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Identification of markers of squamous cell carcinoma in sarco/endoplasmic reticulum Ca^{2+} ATPase isoform 2 heterozygote mice keratinocytes with altered Ca^{2+} signaling

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실험실에 와서 어리보기 였던 제가 이제는 어느새 마님이 되었네요. 생리학, 약리학 식구들과 보낸 지난 시간들이 새록새록 합니다. 어려운 연구비처리에 머리에 쥐나지만 똘똘한 포토제닉 보령, 생리방 최장신이자 미소가 예쁜 아란공주, 언제나 발랄하고 늘씬한 간지효진, 박사과정에 도전한 동갑내기 정미, 유민이를 씩씩하게 키울 늘 분주한 양유미 선생, 곧 예쁜 아가엄마가 되지만 여전히 스타일리쉬한 수영, 항상 밝게 호응해주는 친절소영, 웃음이 시원한 멋쟁이 막내보화까지 다들 너무나 고맙습니다. 그리고, 이미 졸업한 우리 식구들, 미국에서 열심히 포닥하는 민석이, 본 논문의 일등공신인 작은 거인이자 새색시 해미, 착한 승혜, 컴맹인 제게 많은 도움을 준 새내기 사회인 조해군, 그리고 저의 귀찮은 요구에도 열심히 따라준 경은이와 정연이, 실험하는데 많은 도움을 준 조직학 교실의 경원, 민정, 같이 수업들어 좋았던 해부학 교실 미선, 어리지만 든든한 언니 같던 재희, 그리고 프로그램 선생님이신 조장기 선생님, 샌프란시스코에서

포닥생활을 만끽하고 계실 네오팜의 염종경 차장님께 고마움을 전합니다. 이외에도 너무나 많은 분들에게 사랑을 받았습니다. 일일이 언급하지는 못하지만 다들 너무나 고맙습니다.

또한, 제가 이렇게 건강하고 올바르게 성장할 수 있도록 해주신 부모님께 마음을 다해 감사 드립니다. 늦게 시작한 공부라서 준비과정이 필요한 제게 그럴 때마다 용기를 주시던, 존재만으로도 제게 힘이 되시는 부모님, 이제는 연로하신데도 전 공부하느라 제대로 해드린 것도 없습니다. 든든한 아군으로 버팀목으로 그 자리에 계셔주세요. 언제나 사랑해요. 엄마 아빠. 여전히 공부하느라 큰언니, 큰누나 노릇 제대로 못하지만, 언제나 잘 따라주던 내 동생들, 분위기 메이커 연정, 순하고 착한 수경, 근육맨 막내 지송, 너희들 너무나 사랑한다.

어느새 제 옆자리에 와서 제 가슴을 꽉 채워버린 제 반쪽에게 감사해요. 학위과정이라고 공부한다고 많은 신경도 쓰지 못했는데, 여러모로 부족한 제게 변함없이 아껴주고 이뻐해 줘서 너무너무 고마워요. 가끔 이런 행복을 누리는 게 얼마나 큰 행운인지 모른다는 생각을 합니다. 박사를 마치고 포닥을 하러 외국으로 가게 되더라도 아빠로, 오빠로, 친구이자 연인으로 함께 할 수 있어서 기뻐요. 무엇보다 저도 변함없이 곁에서 든든한 당신의 지원군이 될 수 있어 행복합니다.

마지막으로, 전 그분께 너무나 많은 관심과 사랑을 받았습니다. 처음엔 제 곁에 안 계신 줄로만 알았습니다. 이제는 아버지 하나님의 소중한 자녀가 되었지요. 제가 많은 어려운 일들을 극복할 수 있도록, 그래서 제 삶에 축복의 날개를 주시고 훨훨 세상을 날아 볼 수 있게 해주시고 세상에 나아가서 더 많은 누림이라는 기회를 주신 하나님 아버지께 온 마음을 다해 감사 드립니다.

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홍정희 드림

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Abbreviations

SERCA2: sarco/endoplasmic reticulum Ca^{2+}-ATPase 2

SCC: squamous cell carcinoma

$[Ca^{2+}]_i$: intracellular concentration of calcium

TGFβ: transforming growth factor β

PSS: physiologic salt solution

DEGs: differentially expressed genes

MAPKs: mitogen-activated protein kinases

JNK: c-Jun-NH_2-terminal kinase

ERK: extracellular signal-regulated kinase

SOC: store-operated Ca^{2+} influx

Tg: thapsigargin

PLC γ: phospholipase C γ

NFI-B: nuclear factor I-B

PDGFβ: platelet-derived growth factor β

IL: interleukin

ABSTRACT

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A mutation of *Atp2a2* gene encoding the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) causes a skin disorder such as Darier's disease in human and null mutation in one copy of *Atp2a2* leads to a high incidence of squamous cell carcinoma (SCC) in an animal model of mouse. However, a precise mechanism between SERCA2 gene and mode of SCC susceptibility is not clear. In this study, I investigated Ca^{2+} signaling and differential gene

expression in primary cultured keratinocytes from SERCA2 heterozygote (SERCA2$^{+/-}$) mice. SERCA2$^{+/-}$ keratinocytes showed reduced initial increases in intracellular concentration of calcium ($[Ca^{2+}]_i$) and and decreased $[Ca^{2+}]_i$ reduction rate in response to ATP, a G-protein coupled receptor agonist compare to wild type kerationcytes. Higher Ca^{2+} entry was maintained in SERCA2$^{+/-}$ keratinocytes after treatment with thapsigargin, an inhibitor of SERCA pump, than wild type keratinocytes. Protein expressions of plasma membrane Ca^{2+} ATPases, NFATc1, phosphorylated ERK, JNK, and phospholipase γ1 were increased in SERCA2$^{+/-}$ keratinocytes. Using the gene fishing system, I found that gene level of tumor-associated calcium signal transducer 1, thymosin β4, crystalline αB, nuclear factor I-B, procollagen XVIII α1, and mouse porcupine A mRNA were increased. Finally expressions of keratinocyte differentiation-related genes, involucrin, and dermokine β were decreased in SERCA2$^{+/-}$ keratinocytes. These results suggest that SERCA2 haploinsufficiency are related to perturb intracellular Ca^{2+} level through the alterations of Ca^{2+} signaling-related genes and proteins and alternates the gene expression of tumor induction and keratinocytes differentiation.

Keywords: SERCA2, squamous cell carcinoma, keratinocyte, Ca^{2+} signaling, Cancer-related genes, differentiation

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I. INTRODUCTION

Skin cancer is the most common type of human cancer that can metastasize and lead to death. Squamous Cell Carcinomas (SCCs), which arise in multilayered epithelia such as the epidermis, cervix, lip, tongue, and floor of the mouth (Janes *et al*, 2006), are the most common type of oral

cancers and more than 90% of the reported SCCs are malignant (Chen *et al*, 2004).

It has been suggested that SCCs of the skin increase through a variety of process that involve activation of proto-oncogenes and/or inactivation of tumor suppressor genes, ultraviolet irradiation (UV), UV-induced oxidative stress (Kubo *et al*, 2002; Melnikova *et al*, 2005), inflammation (Li *et al*, 2006), DNA damage (Berhane *et al*, 2002), and p53-gene instability (Brash *et al*, 1991; Ide *et al*, 2003). p53 heterozygosity was found to be correlated with tumor induction in a mouse model, which suggests that p53 mutation is an early event in UVB-induced skin carcinogenesis (Rebel *et al*, 2001). Loss of transforming growth factor β (TGF β) type II receptor and the overexpression of K-or H-ras have been shown to induce SCC of the head and neck (Lu *et al*, 2006). In a study evaluating chemical carcinogenesis in mice, topical 9,10-dimethylbenzanthracene and phorbol 12-myristate 13-acetate was found to induce papilloma formation that subsequently progress to carcinoma (Allen *at al*, 2003). Thapsigargin (Tg), a specific inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Hakii *et al*, 1986) pump, and 12-*O*-Tetradecanoylphorbol-13-acetate (Mufson *et al*, 1979) have been identified as tumor promoters that alter the growth and differentiation of mouse epidermis (Lowry *et al*, 1996). Notably, the loss of one copy of the *Atp2a2* allele encoding SERCA2 induced the formation of SCCs in SERCA2 heterozygote

($SERCA2^{+/-}$) mice (Liu *et al*, 2001). Finally, $SERCA2^{+/-}$ mice show enhanced tumor susceptibility that is followed by tumor initiation and progression via elevated expression of wild type H-ras, K-ras, and p53 in $SERCA2^{+/-}$ (Prasad *et al*, 2005), which suggests that aberrant Ca^{2+} signaling due to SERCA2 haploinsufficiency is associated with susceptibility to carcinogenesis.

The Ca^{2+} signal evoked by stimulation of plasma membrane receptors induces activation of phospholipase C (PLC) that hydrolyses phosphoinositide bisphosphate to generate inositol 1,4,5-trisphosphate (IP_3), which triggers the release of Ca^{2+} from intracellular Ca^{2+} stores (Berridge *et al*, 1998). The release of Ca^{2+} from the ER is followed by activation of store operated Ca^{2+} influx channels, which increase the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Berridge *et al*, 1998). Cells then use Ca^{2+}-ATPase pumps, such as SERCA and plasma membrane Ca^{2+} ATPases (PMCAs) pumps to remove Ca^{2+} from the cytosol to establish a higher steady-state Ca^{2+}. Upon termination of the stimulus, SERCA2 reloads the ER with Ca^{2+} to prepare the cells for a next round of stimulation. SERCA also reloads the stores with Ca^{2+} in the periods between Ca^{2+} spikes during Ca^{2+} oscillations (Shin *et al*, 2001).

SERCA2 is an endoplasmic reticulum-resident protein that plays a critical role in the regulation of Ca^{2+} homeostasis. The SERCA2 pumps have two primary isoforms, SERCA2a and SERCA2b (Lytton *et al*, 1988). SERCA2a is the main isoform expressed in cardiac muscle, whereas SERCA2b is the

house-keeping, ubiquitous ER/SR pump found in all cells. Life is incompatible without SERCA2, as evident from the lack of generation of $SERCA2^{-/-}$ embryos from mating of the heterozygotes (Periasamy *et al*, 1999).

The SERCA2 pumps are responsible for loading the ER with Ca^{2+} and maintaining the Ca^{2+} gradients between the cytosol and the lumen of the ER (Gunteski-Hamblin *et al*, 1988). In addition to its role in Ca^{2+} signaling, ER Ca^{2+} also control several cellular activities, in particular protein folding and the unfolding protein response (Ron and Walter, 2007). Several cell types, including epidermal cells, are particularly sensitive to reduction in SERCA2 activity and ER stress. It has been suggested that the ER Ca^{2+} content contributes to the maintenance and the differentiated epidermis, including skin barrier function (Bikle *et al*, 2001; Elias *et al*, 2002).

Loss of ER Ca^{2+} causes increased cell proliferation and decreased expression of differentiation markers, such as involucrin, filaggrin, and loricrin (Bikle *et al*, 2001). In addition, defective Ca^{2+} homeostasis causes skin diseases such as Darier's disease (DD) (Sakuntabhai *et al*, 1999; Zhao *et al*, 2001; Ahn *et al*, 2003; Foggia, 2004), which is an autosomal dominant skin disorder characterized by multiple keratotic papules caused by a null mutation in one copy of the *Atp2a2b* gene (Sakuntabhai *et al*, 1999). The SERCA2b isoform is abundantly expressed in keratinocytes (Pani *et al*, 2006) and inhibition of SERCA2 with Tg disrupts a variety of biological processes,

particularly during terminal differentiation of keratinocytes (Lowry *et al*, 1996). However, the exact role of SERCA2b in epidermal cells Ca^{2+} signaling and its direct relation to SCC are not known. In the present work, I took advantage of the $SERCA2^{+/-}$ mice to address these questions.

$SERCA2^{+/-}$ mice serve as an animal model for skin tumors and allowed us to study the effect of altered SERCA2 expression on Ca^{2+} signaling in primary cultured keratinocytes as well as expression of tumorigenic factors associated with aberrant SERCA2 function in an attempt to assess the role that the signaling pathway plays in the development of SCC.

II. MATERIALS AND METHODS

1. Animals

Wild-type (WT) and SERCA2$^{+/-}$ mice in a Black Swiss background were generated as described (Periasamy *et al*, 1999), and housed with free access to food and water in a temperature-controlled room (23 ± 1℃) under artificial illumination (lights on 06:00 h ~ 18:00 h) and 55% relative humidity. All animal protocols were performed according to institutional guidelines of Yonsei University College of Dentistry. Genotypes were determined by PCR analysis of tail DNA as described previously (Periasamy *et al*, 1999).

2. Materials

Trypsin, ATP, DNase, Hanks' balanced salt solution (HBSS), trypsin, type I collagen, and collagen were purchased from Sigma; Defined keratinocyte media, gentamycin, penicillin, streptolysin, thapsigargin, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA); fura-2, acetoxymethyl ester (fura-2, AM) was purchased from Teflabs (Austin, TX, USA). All other chemicals were used reagent grade.

3. Primary cultured keratinocyte preparations from WT and

SERCA2$^{+/-}$

Dorsal and ventral skin of one mouse was used for keratinocytes culture. The skins were carefully shaved off all body hair, stretched, tissue debris was removed carefully with a scrapper, and the skins were floated on 0.5% trypsin in HBSS for 25 min at 37°C. The floated epidermis was carefully separated from the dermis, neutralized by HBSS including 0.05% DNase and 20% fetal bovine serum, and minced with scissors. The suspension was filtrated through autoclaved nylon nets to take off the remaining body hair and tissue fragments. After sedimentation, the epidermal cells were washed with HBSS, centrifuged for 5 min at 1,000 rpm, and re-suspended gently in defined keratinocyte media including 5 μg/ml gentamycin. The cells were cultured on type I collagen-coated dishes in an incubator at 5% CO_2 and 37°C and used at 80% confluency. The media was periodically changed every 2-3 days.

4. Histology

Mouse skins from the lip and genitalia were fixed in 4% paraformaldehyde in phosphate-buffed saline overnight at 4°C, and embedded in paraffin wax. Images of 6 μm thick paraffin sections stained with hematoxylin and eosin (H & E) were obtained using a Leica microscope (Germany).

5. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was determined in primary mouse keratinocytes by fura-2, AM in an extracellular physiologic salt solution (PSS), the composition of which was as follows (mM): 140 NaCl, 5 KCl, 5mM; 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 glucose, titrated to pH 7.4 with NaOH. The osmolarity of the PSS was 310 mOsm. Ca^{2+}-free medium contained 1 mM EDTA and 1 mM ethyleneglycol-bis-(β-aminoethylether)-N, N, N′, N′-tetra acetic acid (EGTA) in PSS. The primary keratinocytes were cultured on collagen-coated cover glasses for measuring $[Ca^{2+}]_i$. The cells were loaded with 3 μM fura-2/AM for 1hr and after washing with standard solution, $[Ca^{2+}]_i$ was measured by alternately illuminating the cells at wavelengths 340 and 380 nm, and the emitted light was passed through a 510 nm cutoff filter and was collected with a CCD camera and analyzed with a MetaFluor system (Universal Imaging Co., Downingtown, PA, USA). The 340/380 fura-2 ratio was taken as a measure of $[Ca^{2+}]_i$ and fluorescence images were obtained at 3 s intervals.

6. Western immunoblotting

Cells were lysed by adding RIPA (radio-immuno precipitation assay) buffer containing in mM; 10 HCl (pH 7.8), 150 NaCl, 1 EDTA, 1% NP-40, 10 Na_3VO_4, 10 NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml

PMSF. Lysates were centrifuged at 11,000 × rpm for 10 min, and supernatants were collected for immunoblotting. Protein concentration was determined using the BCA assay kit (Pierce, Rockford, IL, USA). Equal protein amounts (40 μg) were separated by 10% SDS-PAGE gel (Bio-Rad, CA, USA) and electro-transferred onto nitrocellulose membranes. The membranes were then incubated in 5% skim milk powder in TBST (mM); 20 Tris-HCl, (pH 7.6), 137 NaCl, and 0.1% Tween 20 for 1hr, and incubated sequentially with primary antibody and followed by horseradish peroxidase-conjugated secondary antibody (SantaCruz Inc., SantaCruz, CA, USA). Blotted proteins were visualized by an enhanced chemiluminescence reagent (IntRON, South Korea).

7. First strand cDNA Synthesis

Total RNA was extracted from cultured keratinocytes and was used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42℃ in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5 × reaction buffer (Promega, Madison, WI, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM dT-ACP1 (5’-CTGTGAATGCTGCGACTACGATIIIIIT-3’), 0.5 μl of RNasin® RNase Inhibitor (Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (Promega). First strand cDNAs were diluted by the

addition of 80 μl of purified water for RT-PCR.

8. ACP-based GeneFishing™ PCR for second strand synthesis

Differentially expressed genes (DEGs) were screened by ACP-based PCR method (Kim *et al*, 2004) using the GeneFishing™ DEG kits (Seegene, South Korea). The PCR for second strand synthesis was performed according to the manufacturer's protocol. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide. Expression levels of DEGs were calculated by the MetaMorph system (Universal Imaging Co., Downingtown, PA, USA).

9. Direct sequencing

The bands of the DEGs were re-amplified and extracted from the gel using the GENCLEAN®II Kit (Q-BIO gene, CA, USA), and directly sequenced with ABI PRISM®3100-*Avant*Genetic Analyzer (Applied Biosystems, CA, USA).

10. Data analysis and Statistics

All data were given as mean ± SE. Statistical significances of between groups were determined using the Student's t-test.

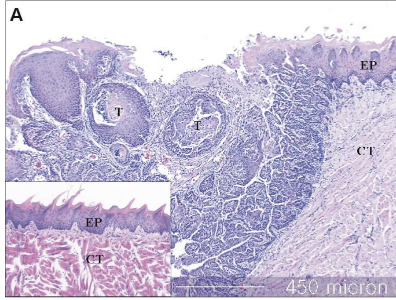
III. RESULTS

1. Development of squamous cell carcinoma in $SERCA2^{+/-}$ mice

Cancer, which was first observed in 22-week old $SERCA2^{+/-}$ mice, reached 100% incidence by the time the mice were 52-week of age. Skin samples were removed from the lips, tongues, and prolapsed perineum of 52-week-old $SERCA2^{+/-}$ mice and analyzed by H & E staining. A comparison of these samples with samples obtained from wild type mice revealed that the lesions on the tongue were composed of tumor cell nests that had invaded the connective tissues (WT, insert) (Fig. 1, A). In addition, tissue samples obtained from exposed skin exhibited the highest frequency of carcinoma. Furthermore, the skin lesions on the lip and perineum showed sheets of invasive squamous cells that lacked an architectural pattern with frequent central necrosis (Fig. 1, B and C). Observing the lesions under high-power revealed the presence of common features of cellular abnormalities such as pleomorphism, hyperchromatism, frequent mitosis, and aberrant accumulations of keratin (Fig. 1, D-F).

2. Expressions of $[Ca^{2+}]_i$ signaling proteins in $SERCA2^{+/-}$ keratinocytes

Western blot analysis was performed on keratinocyte lysates obtained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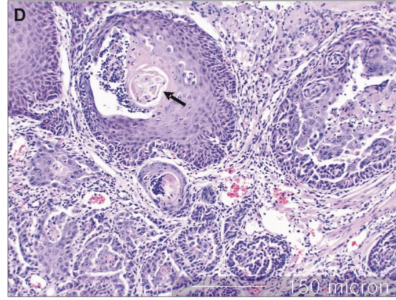
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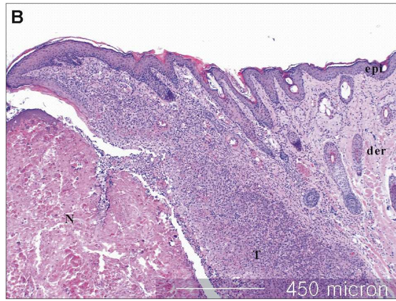
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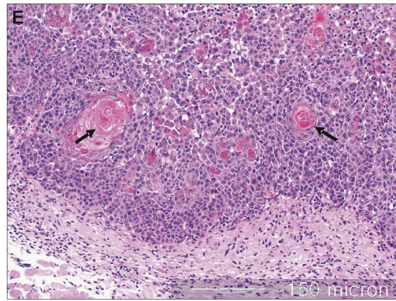
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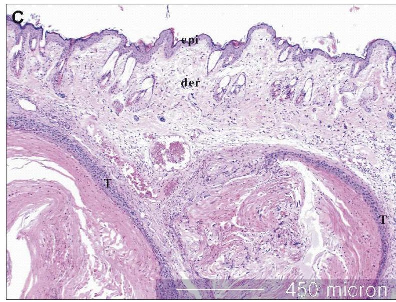
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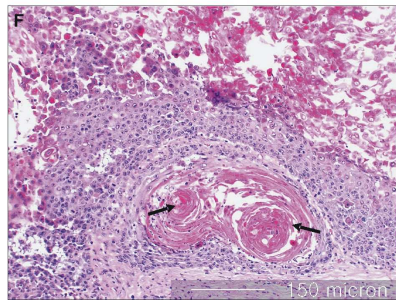
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Fig. 1. Squamous cell carcinomas in the tongue and skins of 52-week-old SERCA2$^{+/-}$ mice. A, The tongue lesions are composed of tumor cell nests (T) invading the connective tissues (CT). Example of wild type tissue is provided in the insert (H & E, original magnification ×40). B-C, The skin lesions of lip and perineum (H & E, original magnification ×40). D-F, High-power view of tongue, lip, and perineum lesions (H & E, original magnification ×100). T: tumor cells, EP: normal epithelium of the tongue. CT: underlying connective tissues, epi: epidermis, der: dermis, N: necrosis, Keratin (arrow)

from WT and SERCA2$^{+/-}$ mice to test for adaptive expression of Ca^{2+} signaling proteins caused by a partial loss of SERCA2. All analyses were conducted on mice that were more than 6 weeks of age to ensure that the epidermal layer was sufficiently mature. Figs. 2A and 2B show that the level of SERCA2b protein was 31.2 ± 1.1% lower in SERCA2$^{+/-}$ than in WT mice (n=4). As I found before in brain extracts (Zhao *et al*, 2001), reduced expression of SERCA2b resulted in up-regulation of PMCA expression. Densitometric analysis of the results revealed that the level of PMCA protein was 4.54 ± 0.89-fold higher in SERCA2$^{+/-}$ than in WT keratinocytes (2A and B, n=4).

3. Altered Ca^{2+} signaling in SERCA2$^{+/-}$ keratinocytes

The Ca^{2+} signal of keratinocytes obtained from the two mice strains was then evaluated by measuring $[Ca^{2+}]_i$. The agonist-evoked $[Ca^{2+}]_i$ signal was triggered by stimulation of the native P2Y2 receptors with high concentration of ATP (Fig. 3A, n=10). Several parameters of the Ca^{2+} signal were modified in the SERCA2$^{+/-}$ cells. The ATP-induced maximum increase of $[Ca^{2+}]_i$ of cells maintained in media containing 1.0 mM extracellular Ca^{2+} $(Ca^{2+})_o$ is reduced by 79.4 ± 2.1% in SERCA2$^{+/-}$ keratinocytes, as reflected in the reduction in the 340/380 ratio (Fig. 3C, *left panel*). $[Ca^{2+}]_i$ was

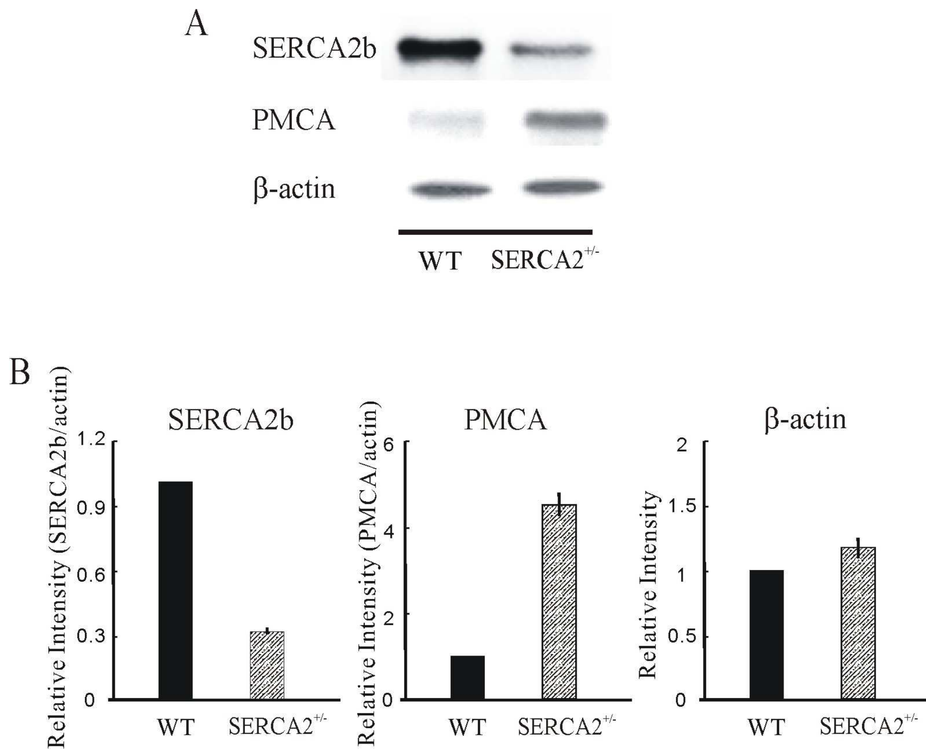
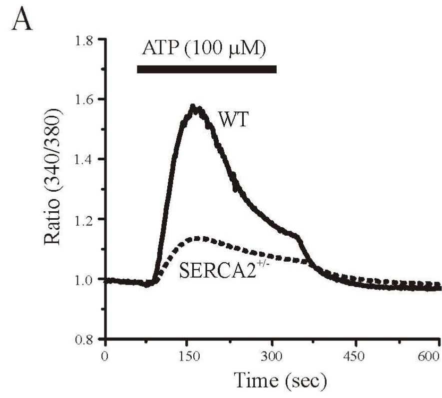


Fig. 2. Expressions of Ca^{2+} signaling proteins in primary keratinocytes from wild type and SERCA2$^{+/-}$ mice. A and B, show protein (40 μg) levels of SERCA2b, PMCA, and β-actin in primary keratinocytes. β-actin was used for immunoblotting control. Results are depicted as mean± S.E.

A



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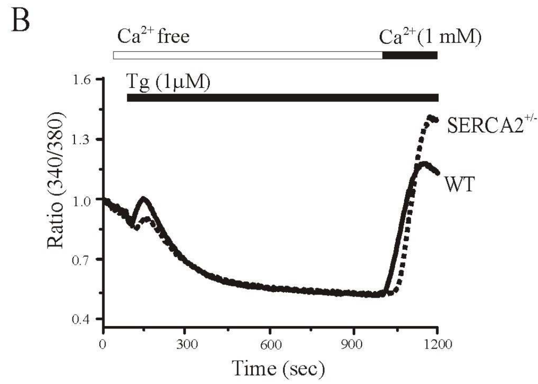

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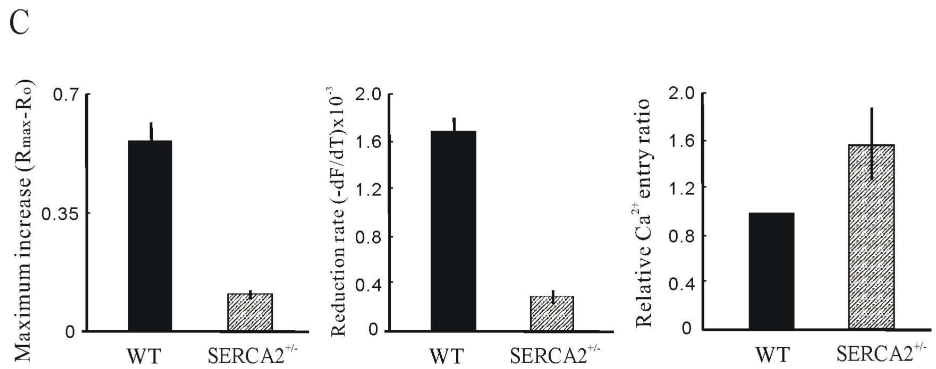

Fig. 3. Altered Ca^{2+} signaling in primary keratinocytes from wild type and $SERCA2^{+/-}$ mice. A, Primary keratinocytes obtained from wild type (WT, solid trace) and $SERCA2^{+/-}$ (dotted trace) mice were stimulated with 100 μM ATP in PSS. B, Cells were perfused with Ca^{2+}-free medium (open bar) and then treated with 1 μM thapsigargin (Tg) in Ca^{2+}-free medium for 15min. After completion of store depletion Ca^{2+} influx was assayed by the addition of 1 mM Ca^{2+} to the perfusion media (marked by dark bar). C, Maximum increase in $[Ca^{2+}]_i$ as revealed by the increase in Fura2 (R_{max}-R_0) ratio in response to ATP stimulation (*left panel*) and reduction rate calculated as the slope (fluorescence/time, -dF/dT) of sustained intensity (*middle panel*). The rate of Ca^{2+} entry obtained from the period of Ca^{2+} addition to cells with depleted stores, as in panel C (*right panel*). Results are depicted as mean±S.E.

reduced more slowly ± in $SERCA2^{+/-}$ keratinocytes than in WT keratinocytes, with the reduction rate of $SERCA2^{+/-}$ keratinocytes being only 69.0±0.11% of that of WT keratinocytes (Fig. 3C, *second*). Store-operated Ca^{2+} influx (SOC) was assayed by passive depletion of the stores by inhibition of the SERCA pumps with Thapsigargin (Tg) and incubating the cells in nominally Ca^{2+}-free medium (Fig. 3B, n=5) for 15min. Tg-triggered Ca^{2+} release was lower by 55.1 ± 0.7% in $SERCA2^{+/-}$ than in WT keratinocytes, probably due to reduced SERCA2 activity and reduced Ca^{2+} content in the stores. Although the rate of Ca^{2+} influx was not statistically different in cells stimulated with ATP, SOC activity was 1.58 ± 0.32-fold higher in $SERCA2^{+/-}$ keratinocytes than in WT keratinocytes (Fig. 3C, *right panel*).

4. Patterns of cancer-related signaling proteins

Tumor induction and development results from activation of a cascade of signaling events that often involves members of the superfamily of mitogen-activated protein kinases (MAPKs), including c-Jun-NH_2-terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK). Remarkably, the steady-state level of the expression of phospho-JNK protein and phospho-ERK protein is increased by 1.37 ± 0.04 and 2.05 ± 0.47-fold in $SERCA2^{+/-}$ keratinocytes, respectively (Fig 4A and B, n=4). NFATc1 activation is related to the level of PLC γ1 phosphorylation and cytosolic Ca^{2+}

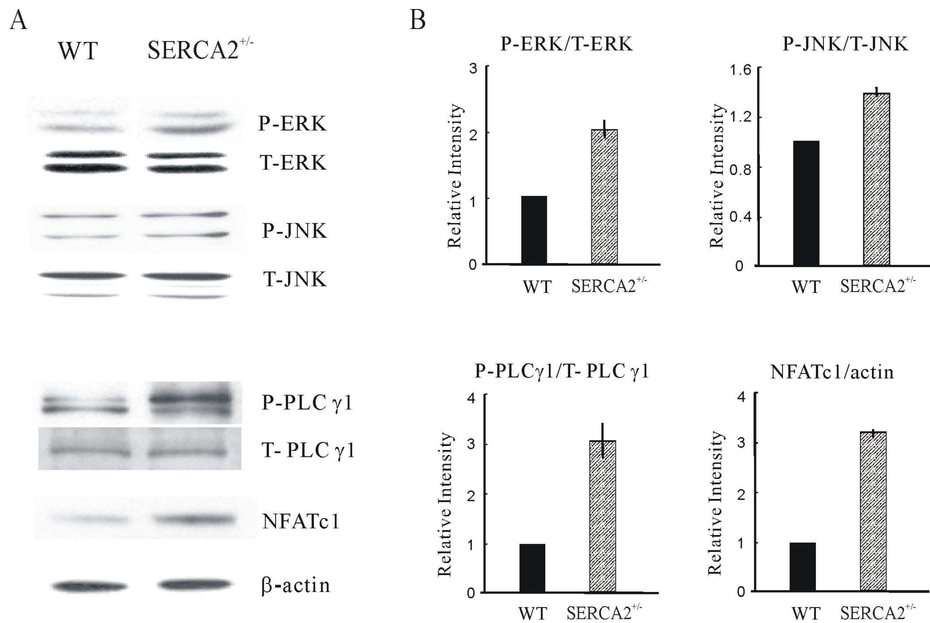

Fig. 4. Effect of disrupted Ca^{2+} signaling on expression of cancer-related signaling proteins. A , show protein level of phospho-ERK (P-ERK), total-ERK (T-ERK), phospho-JNK (P-JNK), total-JNK (T-JNK), phospho-PLC γ1 (P-PLC γ1), total-PLC γ1 (T-PLC γ1), NFATc1, and β-actin in primary cultured keratinocytes. B shows the summary of multiple experiments with n≥4.

level (Hao *et al*, 2003). Therefore, I compared the level of PLC γ1 phosphorylation between the two cell types. Although the level of PLC γ1 is the same in the two cell types, the level of phospho-PLC γ1 protein was 3.06 ± 0.58-fold higher in SERCA2$^{+/-}$ than in WT keratinocytes (Fig 4A and B, n=4). Accordingly, the expression of NFATc1 was 3.24 ± 0.09-fold higher in SERCA2$^{+/-}$ than in WT keratinocytes.

5. Patterns of differentially expressed genes (DEGs) in SERCA2$^{+/-}$ keratinocytes

Increased expression of NFATc1, and probably other transcription factors, is likely to affect the expression of cell cycle and other tumor-related genes. To search for genes affected by the partial deletion of SERCA2, the mRNA pool expressed in 52-week old WT and SERCA2$^{+/-}$ keratinocytes (n=4) was compared using an ACP-based gene fishing PCR method. With this approach I found 9 genes that were differentially expressed between the keratinocytes (Fig. 5). The DEGs that showed the greatest differences were selected for direct sequencing, and the sequences were then evaluated using BLAST searches of the GenBank database (Table 1). Genes that were increased in SERCA2$^{+/-}$ keratinocytes included tumor-associated calcium signal transducer 1, thymosin β4, crystalline αB, nuclear factor I-B (NFI-B), and procollagen XVIII α1, whereas involucrin and dermokine β which are related to

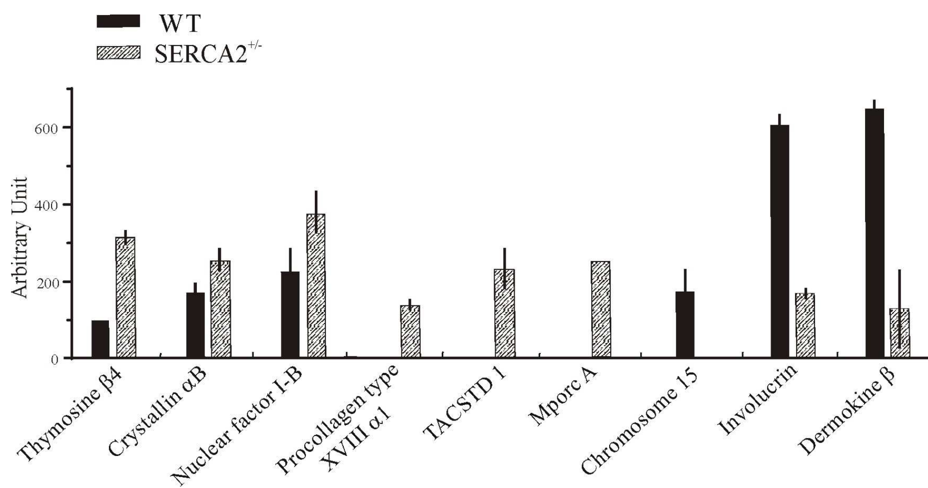

Fig. 5. Effect of disrupted Ca^{2+} signaling on expression of cytokine-related signaling proteins. Relative expression level of ACP-based differentially expressed genes. Results are depicted as mean±S.E

Table 1. Identification of differentially expressed transcripts in response to SERCA2 haploinsufficiency.

A. Up regulation

Identity	Genbank Acc. No.	Base pairs	Sequence Homology
Thymosin β4	BC 018286	603	99% (377/378)
Crystallin αB	BC 010768	848	100% (477/477)
Nuclear factor I-B	BC 096542	2637	100% (596/596)
Procollagen type XVIII α1	BC 064817	5029	100% (585/585)
Tumor-associated calcium transducer 1	BC 005618	1492	100% (575/575)
Mporc A mRNA for porcupine A	AB 036747	1886	99% (416/418)

B. Down regulation

Identity	Genbank Acc. No.	Base pairs	Sequence Homology
Involucrin	NM_008412	1902	99% (369/370)
Dermokine β	AY 622963	1554	100% (441/441)
Chromosome 15	similar	-	-

The percentage expressions are based on BLAST searches of the GenBank databases. The numbers in brackets show the number of bases (query/subject) that were compared.

keratinocyte differentiation, were decreased in SERCA2$^{+/-}$ keratinocytes.

6. Patterns of differentially expressed cytokine-related genes in SERCA2$^{+/-}$ keratinocytes

Cancer cells are capable of attracting different cell types into the tumor environment through angiogenic factors and cytokines. Therefore, to determine which cytokines were involved in the cutaneous carcinogenesis caused by SERCA2 haploinsufficiency, ACP-based cytokine PCR was performed. Fig 6 shows the expression patterns of cytokines in 52-week old WT and SERCA2$^{+/-}$ keratinocytes (n=4). None of the known housekeeping genes analyzed (SDHA; HPRT1, and β2-microglobulin; β2M) was changed in SERCA2$^{+/-}$ keratinocytes. The expression of TGF β1 and 3 as well as platelet-derived growth factor β (PDGF β), interleukin (IL)-11, and IL-16 increased in SERCA2$^{+/-}$ keratinocytes.

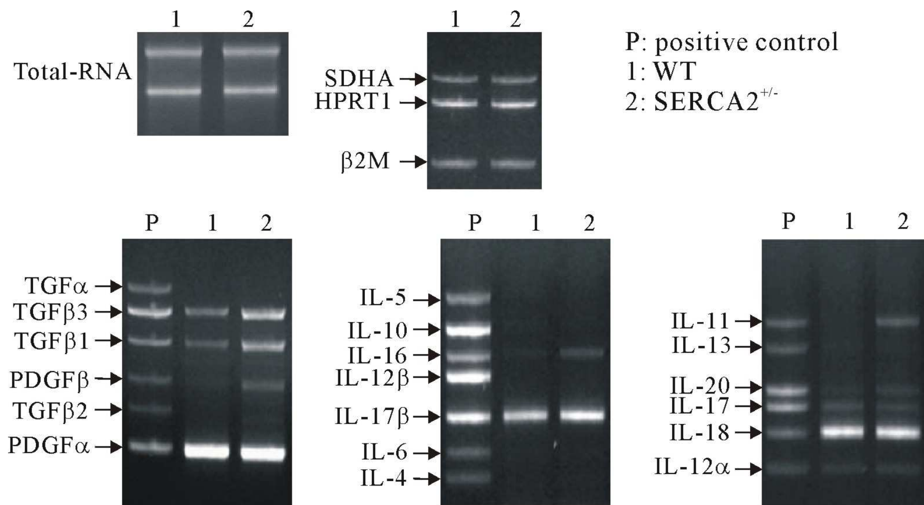

Fig. 6. Patterns of cytokine-related differentially expressed genes in response to SERCA2 haploinsufficiency. mRNA levels of cytokine-related DEGs. P; positive control, 1; WT, 2; $SERCA2^{+/-}$, House keeping genes (SDHA, HPRT1, and β2M) were used as mRNA controls.

IV. DISCUSSION

Evaluating Ca^{2+} signaling in keratinocyte from SERCA2$^{+/-}$ mice suggests a critical role of aberrant Ca^{2+} signaling in carcinogenesis. Reduction in SERCA2.activity increased both SOCs-mediated Ca^{2+} influx and PMCA activity. Similar results have been reported by Pani *et al*, (2006) who concluded that disruption of ER Ca^{2+} content is associated with an increase in TRPC1 expression. In addition, in a previous work I reported that adaptation of Ca^{2+} signaling to partial loss of the SERCA2 pump is accomplished via up-regulation of the PMCA protein and activity (Zhao *et al*, 2001). However, the precise mechanisms by which PMCA and TRPC1 or other SOCs-related proteins such as Orai1 (Smyth et al, 2006; Hewavitharana *et al*, 2007; Cheng *et al* 2008) and STIM1 (Várnai *et al*, 2007; Jousset *et al* 2007) expression is induced in response to SERCA2 haploinsufficiency are not yet known.

Another adaptation of Ca^{2+} signaling protein to disruption of ER Ca^{2+} content discovered in the present work is increased phosphorylation of PLC γ1. This is likely to increase cellular excitability to maintain the Ca^{2+} signaling pathway in a more active state under resting condition. Accordingly, I found that expression of the Ca^{2+}-regulated transcription factor NFATc1 is markedly increased in SERCA2$^{+/-}$ keratinocytes. The NFAT system plays a critical role in cellular hypertrophy and differentiation through interaction with the MAPK

pathways (Crabtree and Olson, 2002; Molkentin, 2004; Gwack *et al*, 2007)

MAPKs such as JNK, ERK, and p38 are important regulatory proteins that transduce various extracellular signals into intracellular events (Seger *et al*, 1995). These MAPKs are modulated by disruption of ER Ca^{2+} homeostasis (Liang *et al*, 2006), and ERK activation is an important downstream effecter mechanism for cellular protection from ER stress (Hung *et al*, 2003). The SERCA level in the ER is critical in determining ER Ca^{2+} content (Papp *et al*, 2004). The results of this study suggest that dysfunction of Ca^{2+} homeostasis caused by a partial loss of SERCA2 in $SERCA2^{+/-}$ karatinocytes is related to events of remodeling of the Ca^{2+} signal-related protein, such as activation of PLC $\gamma 1$, increased SOCs and increased PMCA that increases cell excitability.

Tumor development is a continuous process of mutations accumulation that lead to several intermediate phenotypes as well as the final phases of autonomy, unlimited growth and metastasis (Smilenov, 2006). It was shown SERCA2 haploinsufficiency rather than a loss of heterozygosity is responsible for the cancer phenotype of $SERCA2^{+/-}$ mice with enhanced tumor susceptibility resulting in keratinized epithelia (Prasad *et al*, 2005). In addition, data from human and mouse tumors strongly suggest that heterozygosity leading to haploinsufficiency functionally contributes to tumor development (Smilenov, 2006). For example, haploinsufficiency for adenomatous polyposis coli (*APC*), flap endonuclease 1 (*Fen 1*), breast

cancer-associated gene 1, 2 (*BRCA1, BRCA2*), *p53*, and retinoblastoma (*RB*) have been shown to contribute to tumorigenesis (Santarosa *et al*, 2004). Hence, the haploid form of the *Atp2a2* gene can cause ER-specific stress, which in turn has a permissive effect on tumor development in mouse keratinocytes.

In this study, I employed a new differential displayed ACP-based PCR technique (Kim *et al*, 2004) to compare gene expression in SERCA2$^{+/-}$ and WT keratinocytes. The results of this ACP-based PCR were then used to clarify the mechanism by which tumor development occurs. In terms of increased gene expression, up-regulated DEGs in keratinocytes correspond to a variety of carcinogenesis signals (Table 1A). For example, Tumor-associated Ca^{2+} transducer (TACSTD) is involved in the early stages of human lung adenocarcinogenesis (Shimada *et al*, 2005). The results of this study revealed that TACSTD1 is also involved in carcinogenesis in keratinocytes. It has been suggested that collagen type XVIII expression is a useful prognostic marker in patients with nonsmall cell lung carcinoma (Chang *et al*, 2004). Although the detailed function of procollagen type XVIII and the pre-form of collagen type XVIII are still unknown, their expression in SERCA2$^{+/-}$ keratinocyte provides insight into the mechanism by which carcinogenesis occurs. The NFI-B gene is related to tumor generation via fusion with its translocation partners which include *HMGIC* gene (Gronostajski *et al*, 2000). Although the NFI-B fusion

occupied a small percentage of tumors, aberrant expression of NFI-B NFI-B may play a role in tumorigenesis (Gronostajski *et al*, 2000). Thymosin β4 regulates angiogenesis, which is involved in processes such as wound healing and tumor progression, by controlling the actin cytoskeleton dynamics (Smart *et al*, 2007). Crystallin αB, a small heat shock protein, has chaperone activities in its functional role of holding or folding multiple proteins that have been denatured simultaneously under stress conditions (Chen *et al*, 2004; Ohto-Fujita *et al*, 2007). Finally, porcupine (porc) encodes a multiple transmembrane ER protein that is required for processing Wingless, a family member of Wnt family (Tanaka *et al*, 2000 and 2003) that regulate many developmental functions including carcinogenesis. The expression of Mporc (mouse porcupine) A mRNA may be involved in the development of SCC through Wnt signaling. Although it is unclear if the functions of NFI-B, thymosin β4, crystalline αB, and porcupine A are related to SERCA2 haploinsufficiency, these up-regulated genes may affect carcinogenesis.

Down-regulation of DEGs in keratinocytes corresponds to a variety of signals involved in the differentiation of keratinocytes (Table 1B). For example, Tg-induced depletion of intracellular ER Ca^{2+} stores inhibited trafficking of desmosomal proteins to the cell membrane and perturbed the structural organization of desmoplakin and involucrin, which are differentiation markers of keratinocytes (Jones *et al*, 1994). Although the

biological mechanisms of down-regulated dermokine-β in SERCA2$^{+/-}$ mice keratinocytes are unclear, it has been suggested that disorder of the SERCA pump disrupts keratinocytes differentiation. Use of a SERCA2$^{+/-}$ mouse model is an obvious candidate for studying the mechanism by which lesional keratinocytes are formed. Keratin 1, 10 (K1, 10) and involucrin are normal differentiation markers, however K6 is indicative of enforced proliferation and activation, which generally occurs during wound healing and inflammation (Prasad *et al*, 2005). The results of this study show that SERCA2 haploinsufficiency contributes to the down-regulation of dermokine β and involucrin in SERCA2$^{+/-}$ keratinocytes. In our screen, I also found reductions in similar genes of chromosome 15. Deletions associated with chromosome 15 are thought to be involved in the development and progression of a variety of human solid tumors, including neuroblastoma (Cunsolo *et al*, 2000), papillary serous peritoneal carcinomas (Cass *et al*, 2001) and small cell lung cancer (Kee *et al*, 2003). The present findings provide the base for further studies to determine the molecular mechanisms by which dysfunction of SERCA2 leads to expression of oncogenes.

Considerable complexity underlay the mechanisms by which TGF signaling regulates cancer initiation. The TGF βs are multifunctional cytokines families that play a pivotal role in the maintenance of tissue homeostasis, and TGF β1 over-expression promotes tumor invasion in the

skin. In addition, TGF β1 over-expression in the basal layer of the epidermis and hair follicles causes a severe inflammatory skin disorder and epidermal carcinogenesis (Li *et al*, 2006). IL-11 is a pleiotropic cytokine that exhibits anti-inflammatory properties (Theodore *et al*, 1996), and Fujita *et al* suggested that the inhibition of allogenic lymphocyte reactivity might be due to the direct action of secreted IL-16 (Fujita *et al*, 2000). The results of the present study shows that SERCA2 haploinsufficiency and perturbation of Ca^{2+} signaling are powerful signals that induce abnormal expression of the TGF β1 and IL-11 genes, which are likely to be involved in induction of the observed carcinogenesis.

In conclusion, our findings show that haploinsufficiency of SERCA2 results in reduced intracellular free Ca^{2+} levels and adaptation of the Ca^{2+} signaling machinery to increase cell excitability. The increased cell excitability, in turn induces the switch-on mechanism of oncogenes expression and the switch-off mechanism of keratinocyte differentiation (Fig. 7). Although the precise mechanisms by which cutaneous carcinogenesis occurs remain to be elucidated, the results of this study demonstrate that SERCA2 haploinsufficiency is linked to the promotion of these genetic abnormalities and may be crucial to carcinogenesis, at least in a subset of SCCs.

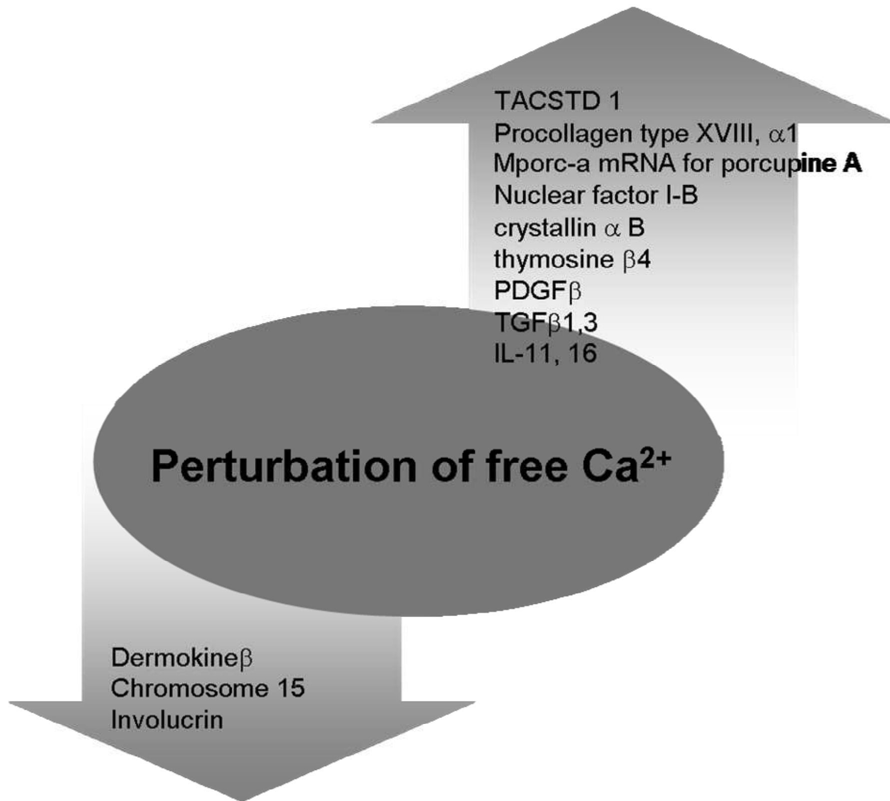

Fig. 7. Schematic diagram of differentially expressed genes (DEGs) in response to SERCA2 haploinsufficiency.

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국문요약

SERCA2 이형접합자 생쥐의 각질형성세포에서 칼슘신호 변동과 연관된 편평상피종 연관 표지자 규명

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근소포체/소포체 칼슘펌프 제 2아형 (SERCA2) 단백을 전사하는 *Atp2a2* 유전자 돌연변이 시 인간에서는 Darier Disease와 같은 피부질환이 발생하고 생쥐동물 모델에서 *Atp2a2* 유전자의 반수체가 결여되면 편평상피종이 유발된다. 그러나 SERCA2 유전자와 편평상피종의 감수성 형태 사이의 정확한 기전은 명확하지 않다. 이에 본 연구에서는 SERCA2 이형접합자 생쥐의 각질형성세포를 일차 배양하여 칼슘신호와 유전자발현의 차이를 조사하였다. SERCA2 이형접합자 각질형성세포는 G 단백연계수용체의 효현제인 ATP 자극에 대해 야생형의 각질형성세포에 비해 세포 내 칼슘농도의 초기증가 정도가 감소되었고 칼슘제거속도가 감소되었다. SERCA 펌프의 저해제인 thapsigargin을 처리한 경우는

야생형의 각질형성세포에 비해 칼슘 유입이 높게 유지되었다. 원형질막 칼슘펌프, NFATc1, 인산화 된 형태의 ERK, JNK, 인지질 가수분해효소인 phospholipase Cγ 제1아형의 단백 발현이 SERCA2 이형접합자 생쥐의 각질형성세포에서 증가됨을 확인하였다. 두 개체에서 유전자 발현 변화를 보이는 유전자 검색법을 통해 SERCA2 이형접합자 생쥐의 각질형성세포에서 tumor-associated calcium signal transducer 1, thymosin β4, crystalline αB, nuclear factor I-B, procollagen XVIII α1, mouse porcupine A의 발현이 증가되었음을 발견하였다. 또한 각질형성세포의 분화관련 유전자인 involucrin, dermokine β는 감소됨을 확인하였다.

이러한 결과는 SERCA2 반수체 기능부전이 칼슘신호관련 유전자와 단백발현을 조절함으로써 세포 내 칼슘 수준의 교란 및 암유발성 유전자의 발현과 각질형성세포의 분화 조절에 관여하는 것임을 제안한다.

핵심되는 말: SERCA2, 편평상피종, 피부상피세포, 칼슘신호, 암관련 유전자들, 분화