CHAPTER 4

Results

4.1 Immunohistochemical study

4.1.1 Demographic and clinicopathologic characteristics of OSCC cases

Of 34 OSCC cases, there were 17 male and female patients with an average age equal to 66.94 years (43-85). Other clinicopathologic characteristics of 34 OSCC cases are summarized in Table 4.1.

Table 4.1 Clinicopathologic characteristics of OSCC cases

Variable		Number of cases
Histologic grading	Well-differentiated	14
	Moderately-differentiated	12
	Poorly- differentiated	8
Location	Buccal mucosa	10
	Lateral tongue	5
	Gingiva/alveolar mucosa	13
	Labial mucosa	1
	Retromolar pad	1
	Lip vermillion	2
	Mandible	2

4.1.2 ADAM9 expression in OSCC specimens

4.1.2.1 Overexpression of ADAM9 in OSCC specimens

By immunohistochemistry, intense ADAM9 staining was observed in the connective tissue layer of OSCC specimens (Fig. 4.1), particularly at both the cytoplasm and the membrane (yellow and black arrowheads, respectively, in Fig. 4.2) of most

cancer cell nests, compared with weak and diffuse ADAM9 staining in the cytoplasm of normal epithelial cells, especially in the suprabasal cell layers (Figs. 4.1 and 4.2). Note that the intensity of ADAM9 staining in the overlying epithelium of OSCC tissue was weak (bracket in Fig. 4.1), and no staining in OSCC sections was observed in the absence of anti-ADAM9 antibody as a negative control (Fig. 4.1). The median IHC score in the OSCC group was significantly higher than that in the normal group ($p<0.001$; Fig. 4.3). Furthermore, when OSCC tissues were categorized according to their histologic grading and their median IHC scores were compared with that in normal group, a significant overexpression of ADAM9 was found in all three groups of histological grading ($p<0.05$; Fig. 4.4).

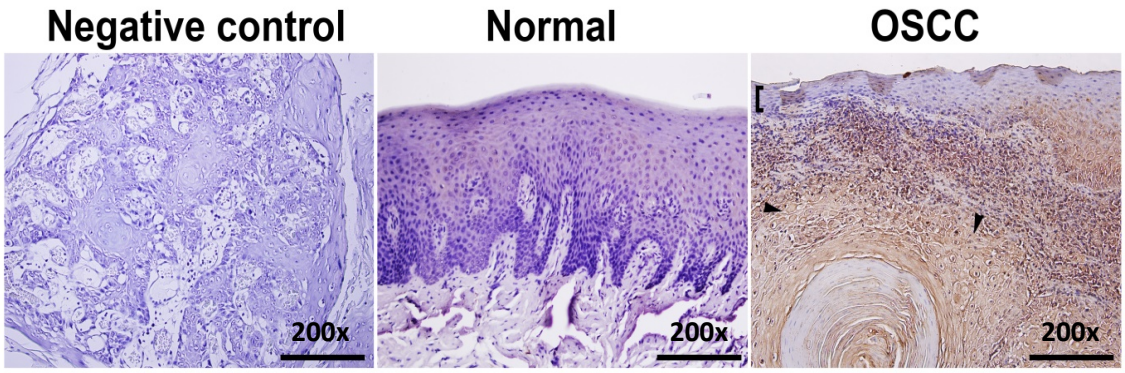

Figure 4.1 Overexpression of ADAM9 in OSCC. A representative image of ADAM9 expression in normal and OSCC tissues is shown. Note weak ADAM9 staining in the suprabasal layers of normal epithelium and in the overlying epithelium of OSCC (bracket), while intense cytoplasmic staining of ADAM9 was observed in tumor cell nests within the connective tissue layer of OSCC (arrowheads). The negative control OSCC section, in which the primary antibody against ADAM9 was omitted, showed no staining. Bars = 200 micron.

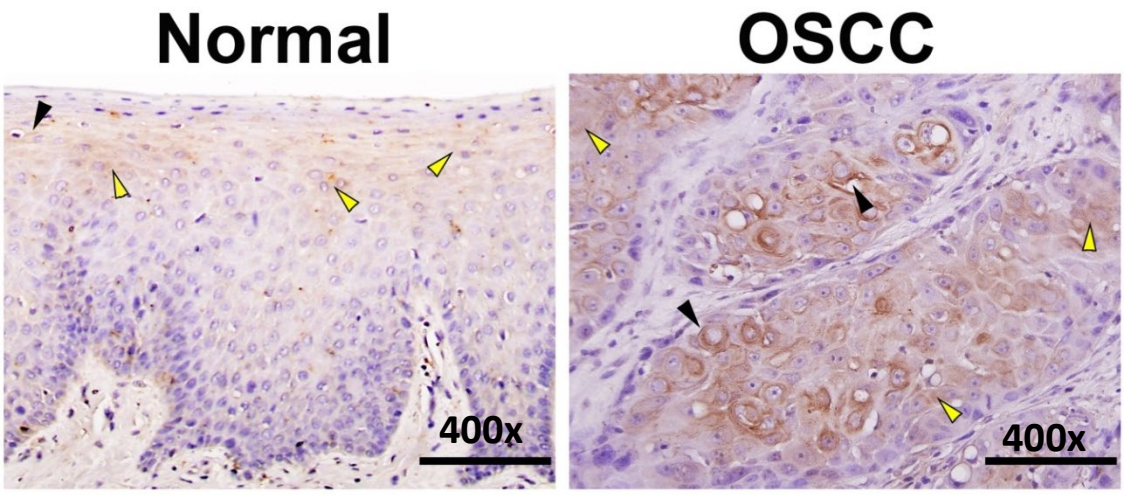

Figure 4.2 ADAM9 expression pattern in OSCC and normal tissues. ADAM9 expression could be observed as cytoplasmic staining (yellow arrowheads) and membrane staining (black arrowheads). In normal tissues, ADAM9 was mainly seen in the suprabasal layers, whereas, in OSCC tissues, ADAM9 staining was found in the tumor islands. Bars = 100 micron.

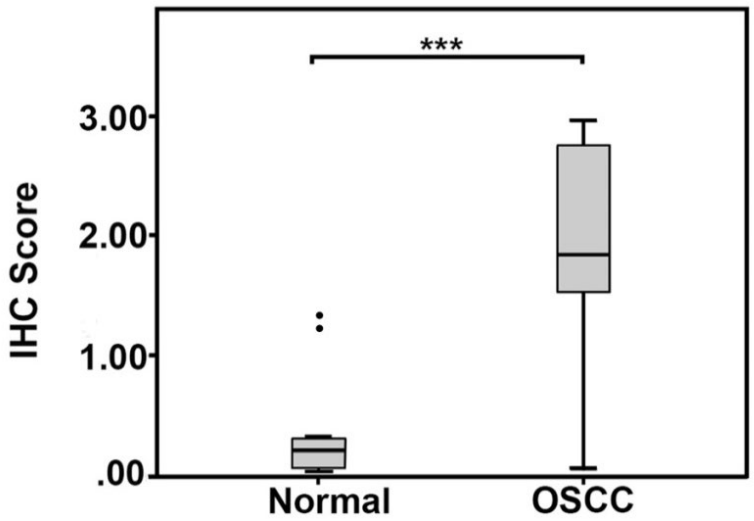

Figure 4.3 A significant ADAM9 overexpression in OSCC tissues. A box plot diagram demonstrated the significantly higher median IHC score for ADAM9 expression in the OSCC group than that in the normal group. *** = $p<0.001$. Two small black circles represent outliers in the normal group.

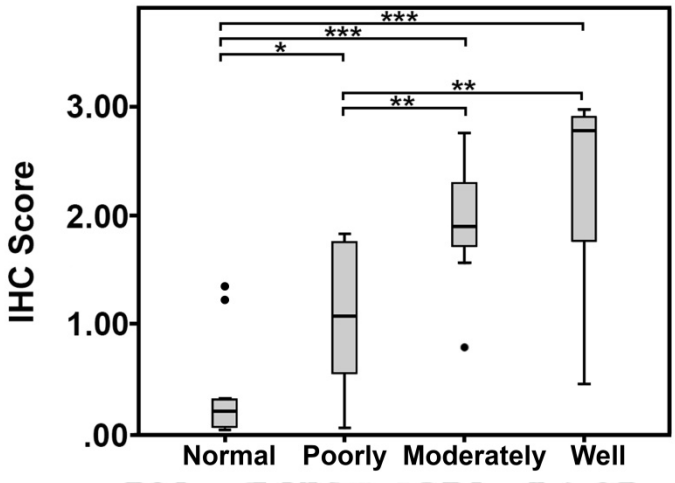

Figure 4.4 A significant overexpression of ADAM9 in poorly-, moderately-, and well-differentiated OSCC. A box plot diagram demonstrated the significantly higher median IHC scores for ADAM9 expression in all histologic grading of OSCC than that in the normal group. Note that the median IHC scores in both moderately- and well-differentiated groups were also significantly higher than that in the poorly-differentiated group. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$. Small black circles represent outliers in the normal and the moderately-differentiated groups.

4.1.2.2 Significantly positive correlation between ADAM9 expression and cancer cell differentiation in OSCC

When all OSCC cases were grouped according to their histologic grading, which included 14 well-differentiated, twelve moderately-differentiated and eight poorly-differentiated cases (Table 4.1), it was found that the intensity of ADAM9 staining was greatest in the well-differentiated OSCC, followed by the moderately- and the poorly-differentiated OSCC, respectively (Fig. 4.5). A semi-quantitative analysis of the staining intensity in all OSCC specimens is summarized in Table 4.2. In general, the intense staining score (=3) was found in more than 60% of the well-differentiated OSCC cases, whereas it was found in only 25% and 0% of the moderately- and the poorly-differentiated OSCC cases, respectively (Table 4.2). On the contrary, more than 60% of the moderately- and the poorly-differentiated OSCC cases were scored as moderate staining (=2). Similarly to the staining intensity score results, the median percentage of positively-stained cells, irrespective of staining intensity, was greater in

the well-(93.24%) and in the moderately-differentiated OSCC (90.05%) than in the poorly-differentiated OSCC (67.60%). By the Spearman correlation test, the IHC scores were positively correlated with an increasing degree of cell differentiation (r=0.557, p=0.001; Fig. 4.6). In other words, the median IHC scores in both moderately- and well-differentiated OSCC were significantly higher than that in the poorly-differentiated OSCC (p<0.01; Fig. 4.6). However, there was no significant difference in the median IHC score between the well- and the moderately-differentiated OSCC (Fig. 4.6).

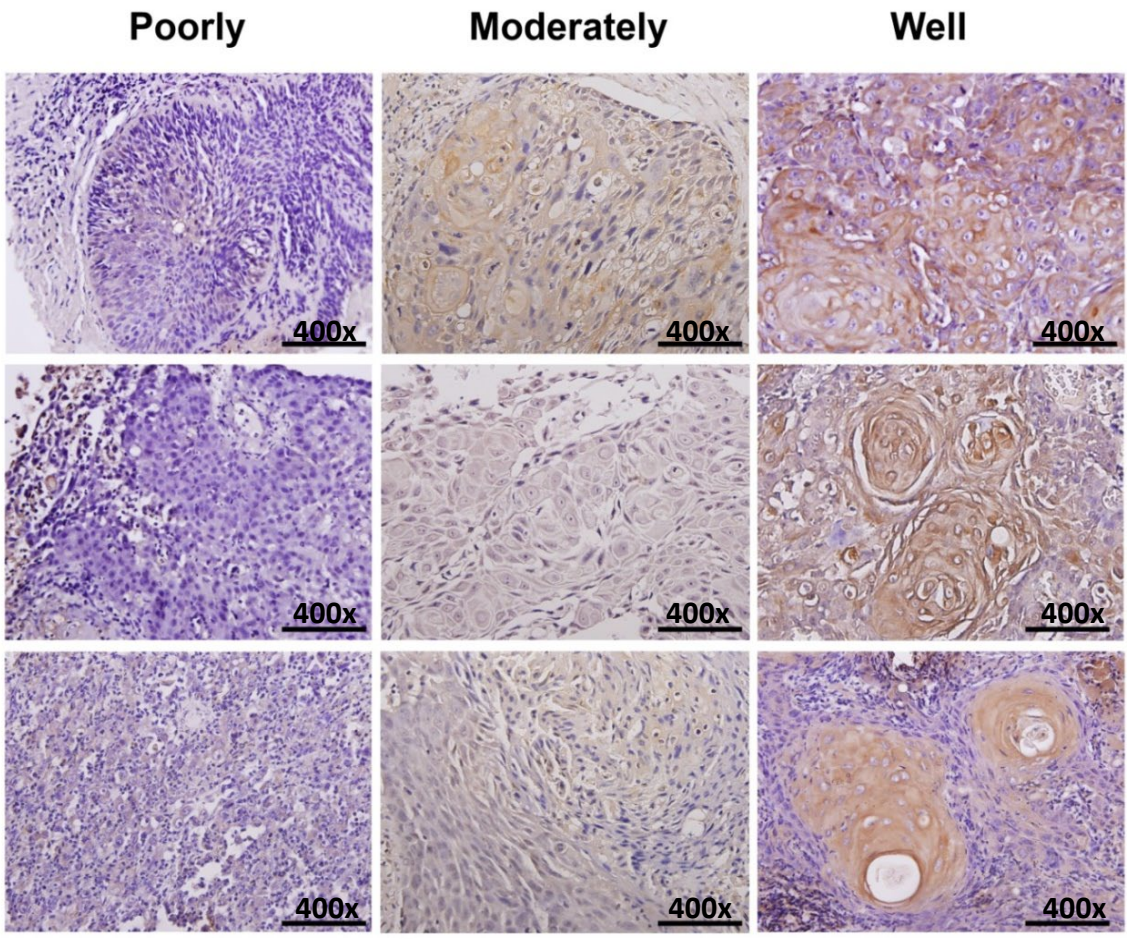

Figure 4.5 Increased ADAM9 expression in relation to cancer cell differentiation. Three representative images of each histologic grading for ADAM9 immunostaining are illustrated. Note the strongest intensity of ADAM9 staining in the well-differentiated OSCC, followed by the moderately- and the poorly-differentiated OSCC. Bars = 100 micron.

Table 4.2 A semi-quantitative analysis of the intensity score for ADAM9 expression in different histologic grading of OSCC

Intensity score [a]	Well-differentiated *N* (%)	Moderately-differentiated *N* (%)	Poorly-differentiated *N* (%)
0	0 (0)	0 (0)	0 (0)
1	2 (14.3)	1 (8.3)	3 (37.5)
2	3 (21.4)	8 (66.7)	5 (62.5)
3	9 (64.3)	3 (25)	0 (0)
Total	14 (100)	12 (100)	8 (100)

[a] scores: 0 = no staining; 1 = weak (light brown staining, visible only with high magnification); 2 = moderate (between 1 and 3); 3 = intense (dark brown staining, visible with low magnification).[56]

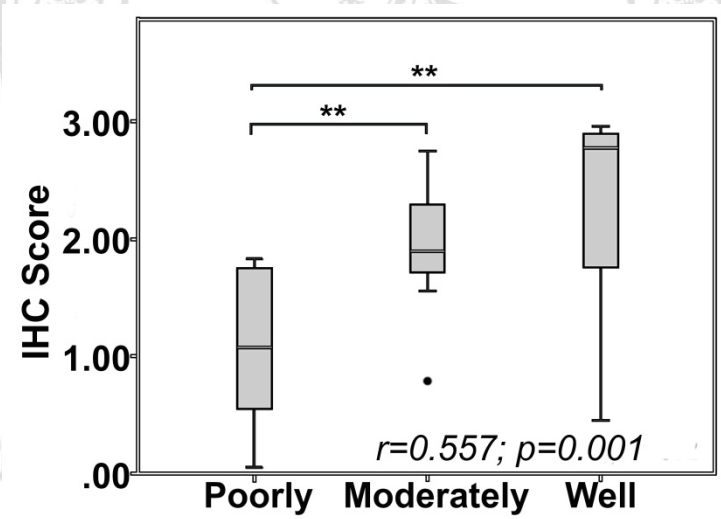

Figure 4.6 A positive correlation between ADAM9 expression and cancer cell differentiation in OSCC. A box plot diagram showed the positive correlation between increased IHC scores (0─3) for ADAM9 expression and enhanced levels of cell differentiation. ** = $p<0.01$. A small black circle represents an outlier in the moderately-differentiated OSCC.

4.2 *In vitro* studies

4.2.1 Expression of membrane ADAM9 in some oral cancer cell lines

First, the expression of membrane ADAM9 in four different oral cancer cell lines, including HN5, HN6, HN15 and HN008, was determined by flow cytometry. It was found that membrane ADAM9 was expressed in three of the four tested oral cancer cell lines, HN6, HN15 and HN008, whereas membrane ADAM9 was not expressed in HN5 (Fig. 4.7). As expected, membrane ADAM9 was expressed in HepG2 as a positive control cell for ADAM9 expression. No expression signal was detected in all tested cell lines, incubated with the purified rabbit immunoglobulins or without the anti-ADAM9 antibody as a conjugate control (Fig. 4.7)

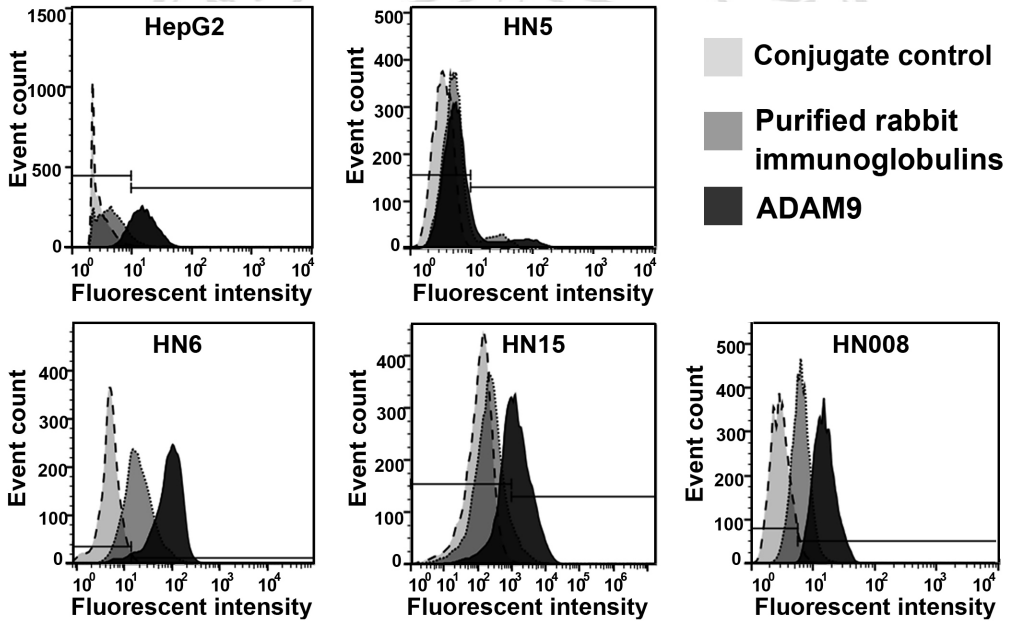

Figure 4.7 Expression of membrane ADAM9 in oral cancer cell lines. A representative histogram for expression of membrane ADAM9 in four oral cancer cell lines, including HN5, HN6, HN15 and HN008, from three separate experiments is shown. Note the expression of membrane ADAM9 (black area) in HN6, HN15 and HN008, whereas membrane ADAM9 expression was not detected in HN5. Membrane ADAM9 was expressed in HepG2, a hepatocellular carcinoma cell line, as a positive control, while there was no membrane ADAM9 expression in conjugate control (light gray area) and

in purified rabbit immunoglobulins (dark gray area) as two negative controls. An *x* axis represents the fluorescent intensity, while a *y* axis represents the event count.

4.2.2 A significant increase in the expression of active ADAM9 in oral cancer cell lines

Varying degrees of ADAM9 expression for the proform, previously reported at 110 or 120 kDa, and for the active form at 84 kDa were detected in the whole cell lysates of HN5, HN6, HN15, HN008, and eight independent HOK cell lines (Fig. 4.8). In general, the expression of active ADAM9 in HN6, HN15, and HN008 was greater than that in HOKs, whereas the expression of the active ADAM9 in HN5 was less than that in HOKs (Fig. 4.8), which was consistent with no fluorescent signal of membrane ADAM9 observed in HN5 (Fig. 4.7). Expression of β-actin was equivalent among different samples (Fig. 4.8). By densitometry, the average percentages of active ADAM9 expression in HN6 and HN008 were significantly higher than that in eight independent HOKs ($p<0.05$ and $p<0.001$, respectively); however, there was no significant increase in the expression of active ADAM9 in HN15 (Fig. 4.9). On the contrary, the mean percentage of active ADAM9 expression in HN5 was significantly lower than that in HOKs ($p<0.001$; Fig. 4.9).

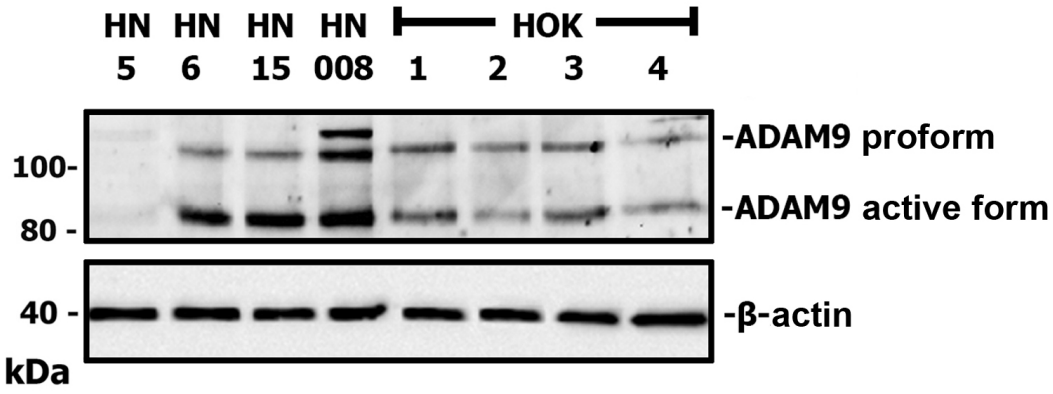

Figure 4.8 Varying degree of ADAM9 expression in each cancer cell line. A representative blot demonstrates varying expression of the ADAM9 proform (110 and 120 kDa) and of its active form (84 kDa) in four oral cancer cell lines, including HN5, HN6, HN15, and HN008, and in four normal human oral keratinocytes (HOK 1─4). Expression of β-actin was equal among all different samples.

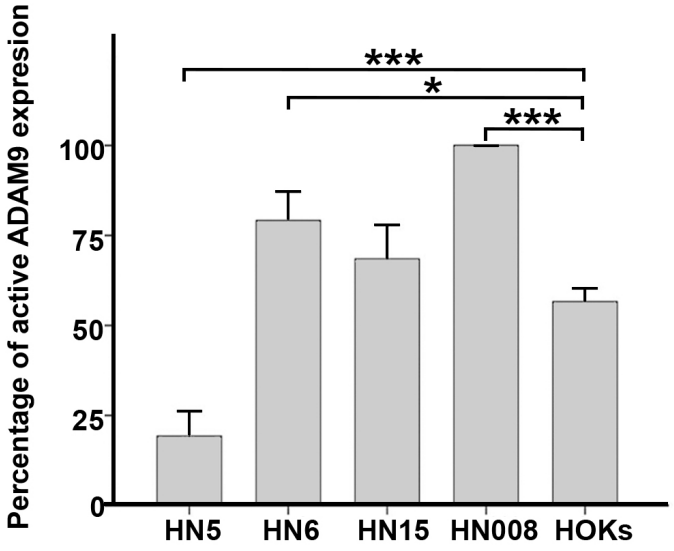

Figure 4.9 A significant increase in ADAM9 expression in some cancer cell lines. A bar graph demonstrates a significant increase in the average percentages of active ADAM9 expression in HN6 and HN008 and a significant decrease in the average percentage in HN5 from five separate experiments (n=5), compared with the average percentage of ADAM9 expression in eight independent HOK cell lines (n=8). The percentage of ADAM9 expression in each oral cancer and HOK cell line was determined by comparison with that in HN008, set to 100%. Error bars = standard deviations; * = p<0.05; *** = p<0.001.

4.2.3 Enhanced ADAM9 expression in differentiated HOKs

Since ADAM9 staining was more intense in well and moderately-differentiated OSCC than in poorly-differentiated OSCC from an immunohistochemical study (Fig. 4.5), reflecting an association between ADAM9 expression and cancer cell differentiation, it was, therefore, interesting to determine the degree of ADAM9 expression *in vitro* in normal differentiated HOKs in comparison with that in normal undifferentiated HOKs. At 80% HOK confluence, serum-free keratinocyte growth medium was switched to low calcium concentration, 0.03 mM, or two high calcium doses, 0.15 and 1.2 mM, for 48 hours, and the degrees of ADAM9 expression in these cells were analyzed by Western blot hybridization. It was shown that expression of both proform (above 100 kDa) and an active form of ADAM9 at 84 kDa was induced by two calcium concentrations, 0.15 and 1.2 mM, compared with a low calcium concentration, 0.03 mM (Fig. 4.10), suggesting that ADAM9 expression is associated with both normal and cancer cell

differentiation. Consistently, the expression of filaggrin, a late differentiation marker, was induced by increased calcium concentrations (Fig. 4.10). Note that β-actin expression was equal among all samples (Fig. 4.10).

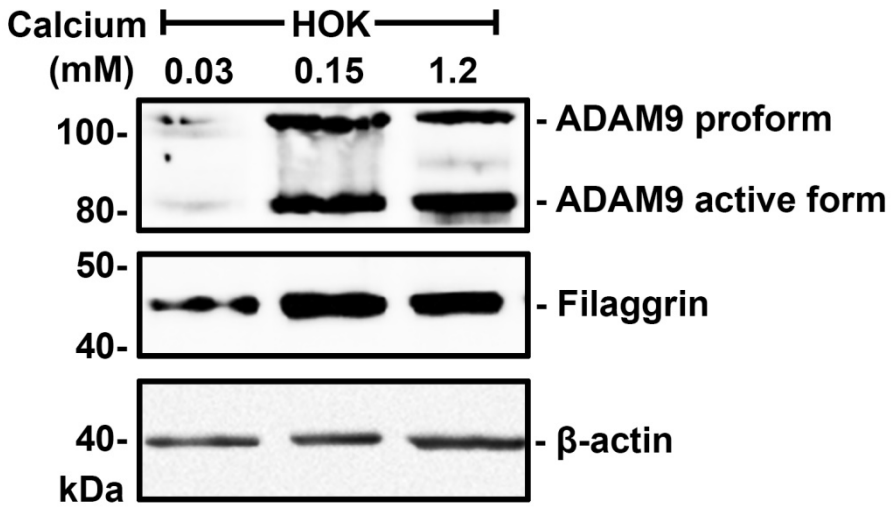

Figure 4.10 Enhanced ADAM9 expression in differentiated HOKs. A representative blot from three independent experiments of HOKs, cultured in three different calcium concentrations, 0.03, 0.15 and 1.2 mM, shows increased expression of both proform (above 100 kDa) and an active form of ADAM9 at 84 kDa in HOKs, cultured in two high calcium doses, 0.15 and 1.2 mM, corresponding to increased expression of filaggrin, a late differentiation marker. Note that β-actin expression was equal among all different samples.