Nkx-2.3 gene in mouse epidermal maturation

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ABSTRACT

In higher vertebrates, from amphibians to humans, epidermal maturation is a conserved developmental process. Using adult epidermal tissue and an established keratinocyte cell line, the mouse Nkx-2.3 homeobox gene was demonstrated, for the first time, to be expressed in mouse epidermal keratinocytes. Under the normal culture condition, the spontaneous aggregation phenomenon, a common initiation step of ES cell differentiation, and the induction of mouse adult K1 keratin, a marker of mature epidermal keratinocytes, were both observed in vitro when the \textit{Xenopus} Nkx-2.3 gene was stably transfected into a mouse pluripotent P19 EC cell line. The induction of mouse K1 keratin by using its \textit{Xenopus} orthologous gene in the mouse P19 cell implies that Nkx-2.3 may play a conserved role in the epidermal maturation of the mouse, as it does in that of the frog (Ma, 2004). However, the CAT assay study on frog adult keratin promoter could not find the induction of adult keratin. This implies there might not be a direct activation of its promoter.

Key terms: Nkx-2.3, epidermis, homeobox gene, skin maturation, differentiation.

INTRODUCTION

Late organogenesis of epidermis bears many conserved features between mammals and frogs. After gastrulation, their skin ectoderms double the layers and change the keratin expression patterns to become embryonic epidermis (Fuchs, 1995), (Jamrich et al., 1987). The bi-layered embryonic epidermis of mammals or frogs is composed of an inner basal layer and an outer apical layer (periderm) (Byrne et al., 1994; Holbrook, 1979; Miller, 1996). Cells of both layers constantly proliferate to accommodate the growth of an embryo or a larva, while some cells in the periderm are sloughed off through programmed cell death (Holbrook and Odland, 1975; Schreiber and Brown, 2003). The embryonic epidermis lasts for a significant period of time, from 36 to 55 days EGA (estimated gestational age) in human embryos (Holbrook and Odland, 1975), from E9.5 to E15.5 in the mouse embryos (Byrne et al., 1994; Fuchs, 1995), and through the stages preceding metamorphosis in tadpoles (Byrne et al., 1994; Fuchs, 1995; Furlow et al., 1997; Holbrook and Odland, 1975). During this period of time, the cell fate of the ectoderm gradually changes into the adult epidermis by expressing adult basal keratins, K5 and K14 in mammals (Fuchs, 1995) or RAK and XAK-C in frogs (Fuchs, 1995; Suzuki et al., 2001; Watanabe et al., 2002). However, these basal cells do not initiate the terminal differentiation program, which stacks up the keratinocytes into multilayer in epidermis until E15.5 of a mouse embryo or stage 61 (the climax of metamorphosis) of a tadpole by expressing suprabasal keratins, K1 and 63 kDa keratin respectively (Fuchs, 1995; Nishikawa et al., 1992; Watanabe et al., 2001; Watanabe et al., 2002). This transition from the appearance of adult basal keratin to the appearance of suprabasal keratin in the developing epidermis is defined in this report as epidermal maturation.

Currently, the mechanism of epidermal maturation is better known in frogs than in mammals. In frogs, it happens during metamorphosis and is triggered by a surge of a single hormone, T3. As T3 level
elevates in blood during metamorphosis, the tadpole basal cells start expressing larval basal keratin, XLK, and later transform into the adult basal cells with a \textit{Xenopus} homologue of mammalian K14 keratin, XAK-C (Watanabe et al., 2001; Watanabe et al., 2002). After committing to the adult fate, the frog basal cells may then begin the terminal differentiation program as $T_3$ reaches the highest level (Miller, 1996; Watanabe et al., 2001; Watanabe et al., 2002). The discoveries of the induction of suprabasal 63 KDa keratin and the precocious transformation of the basal cells into the adult form both in vitro and in vivo by $T_3$ reveal that a $T_3$-mediated mechanism alone is sufficient to initiate epidermal maturation in \textit{Xenopus} (Mathisen and Miller, 1989; Miller, 1996; Nishikawa et al., 1992; Shimizu-Nishikawa and Miller, 1992). In addition to the impact of $T_3$ on keratins, a member of the NK-2 homeobox genes, $XNkx-2.3$, was also found to tightly link with epidermal maturation during metamorphosis by examining the spatial and temporal expression pattern of $XNkx-2.3$ and its response to $T_3$ induction in vivo and in skin explants (Ma, 2004). Although the maturation trigger of mammalian epidermis is still unknown and the action of thyroid hormones (TH) on epidermis is quite different between mammals and frogs (Holt and Marks, 1977; Isseroff et al., 1989), a similar phenomenon of epidermal maturation observed in tadpoles happens in mice, as stated above. Therefore, the expression of $Nkx-2.3$ in the mouse should be explored further.

The $Nkx-2.3$ gene is a \textit{tinman} homologue in the type I NK-2 homeobox gene family, whose role in cardiac mesoderm specification has been well characterized among various species (Harvey, 1996). The gene product contains four functional domains that are well conserved between \textit{Xenopus} and mice (Figure 3). In order to extend the knowledge of $Nkx-2.3$ gene on epidermal maturation from \textit{Xenopus} to mouse keratinocytes, the expression of mouse $Nkx-2.3$ was first investigated in mature keratinocytes in this study. Secondly, to see a conserved role of the $Nkx-2.3$ gene in the epidermal maturation between mouse and frog, the overexpression of the \textit{Xenopus} $Nkx-2.3$ gene to induce the adult K1 keratin in the mouse pluripotent P19 cell line is tested. Finally, to find out the possible relationship between Nkx-2.3 protein and its induced adult keratin, the CAT-reporter system is transiently transfected into various mammalian cell lines.

\textbf{METHODS}

\textit{Collection of mouse tissues}

Newborn mice were produced by mating the super-ovulated female NIHSwiss with male C57BL/6. The epidermis was separated from the dermis after overnight (approximately 18 hours) treatment of skin tissues peeled from newborns, using 1.2 U/ml Dispase II (Roche) at 4°C. The intestine, liver, and heart were also collected for RNA extraction.

\textit{RNA isolation and “same-tube quantitative RT-PCR”}

Total RNA was isolated using the RNaid Plus Kit (Q Biogene). “Same-tube quantitative RT-PCR” was conducted accordingly (Ma, 2004). Briefly, 1-2 $\mu$g of total RNA treated with RNase-free DNase from various tissues was subjected to reverse transcription using random primers (Promega), and one-tenth to one-fifteenth of the product was used for PCR. The PCR was carried out in two steps. First, the solution containing $Nkx-2.3$ primers, cDNA, reaction buffer, and Taq polymerase (Gibco-BRL) was reacted for 8-10 cycles, and then the reaction was stopped at 4°C. In the second step, the internal control ($\beta$-actin) primers and 1X reaction buffer with Taq DNA polymerase were added into the reaction solution for the remaining 30 cycles of amplification. In the case of GAPDH as the internal control gene, the second step of PCR was performed for 25 cycles. Each PCR cycle consisted of 30 sec at 94°C, 30 sec to 1 min at 55°C, and 1.5 min at 72°C. The relative amount of mouse
Nkx-2.3 transcript was determined by calculating the ratio of its band intensity to the band intensity of internal control. The primer pairs used in RT-PCR were MNK23U (5'-TAAGGCCCTGGCGGACACG-3') and MNK23D (5'-CCATGGCCCCCTTTGAAGGAGGAG-3') for the Nkx-2.3 gene, GAPDHU (5'-GTGTTCTACCCCAATGTG-3') and GAPDHD (5'-TGTGAGGAGGATGCTAGTG-3') for the GAPDH gene, and MBU (5'-GGTTCCGATGCCCTGAGGCT-3') and MBD (5'-CCAGGATGGAGCCACCGATC-3') for the β-actin gene; and the expected product sizes were 144 bp, 407 bp, and 279 bp, respectively. The primer set for the mouse K1 gene was MK1U (5'-AGAGGTGGCTCTGGAGGG-3') and MK1L2 (5'-CCATGTTTGGCCTTGATGG-3'), which was expected to produce a 216 bp DNA fragment. The primer set of mouse K1 gene was placed together with the β-actin primers to perform the “same-tube RT-PCR” for 32 cycles.

Plasmid constructs

To prepare a mammalian expression construct, the complete Xenopus Nkx2.3 coding region from pCS2+-XNkx2.3 (gift of Sylvia M. Evans) (Evans et al., 1995) was inserted into a CMV-mediated expression vector, pCDNA4/HisMax (Invitrogen), to make pCDNA4-XNkx2.3. pCDNA4/HisMax contains an N-terminal peptide encoding the Xpress epitope and a polyhistidine tag for detection and purification of the fusion protein. To prepare the promoter-reporter plasmids, a 5.2 kb HindII-HindII DNA fragment from the 5' region of the 63 KDa keratin gene was inserted into pCAT-Basic (Promega) to make pCAT-5200. The shorter 63 KDa keratin promoter constructs, pCAT-2300 and pCAT-630, were constructed by inserting a 2.3 kb HindII-HindII fragment into the HindII linearized pCAT-Basic and by deleting the HindII-BamHI fragment from pCAT-5200 respectively (Figure 6A). Other plasmids that were used included pRSVCAT, a positive control for the CAT assay, and pRSVZ, a lacZ containing plasmid for equalizing the variation of transfection efficiency.

Cell culture and transfection of the XNkx-2.3 gene

The P19 embryonic carcinoma cell line was cultured as described (Skerjanc et al., 1998), except DMEM (Gibco-BRL) was used. 10 μg of pCDNA4-XNkx2.3 was used for CaPO₄ transfection of the XNkx-2.3 gene into P19 cells. Cells were selected in medium containing 400 μg/ml of Zeocin (Invitrogen) for 2 weeks. The drug-resistant clones were picked and maintained in the same medium for another 2-3 weeks to achieve stable growth. The selected clones were routinely cultured in medium containing 200 μg/ml of Zeocin according to the manufacturer's instructions (Invitrogen). The stable lines that expressed high levels of the fusion protein were identified by Western blot analysis using Anti-Xpress antibody (Invitrogen) and further confirmed by immunocytochemical staining. The mammalian cell lines used for transient transfection and CAT assay were HeLa, NIH3T3, HaCaT, and P19. They were maintained in DMEM (Gibco-BRL) with 10% calf serum and incubated at 37°C with 5% CO₂. The CaPO₄ method was also used for transient transfection. Generally, cells were transfected with 8 μg pCAT-630, 10 μg pCAT-2300, or 15 μg pCAT-5200 with 3 μg of pRSVZ plasmid, and 0-20 μg of pCS2+-XNkx2.3 or pCS2+ plasmid. A non-related plasmid, pBluscript KS (Stratagene), was used to equalize the total amount of DNA used for transfection. The cells in 90-mm culture dishes at 30-40% confluence were incubated with the proper plasmids for six hours and subjected to a 90 second 5% glycerol shock. The cells were allowed to express the transfected genes for an additional 48 hours of incubation before being harvested.

CAT Assay

The harvested cells were washed twice with PBS, resuspended in 150 μl of 0.25M Tris-HCl (pH=7.8), and lysed with four quick freeze-and-thaw cycles. Part (20-40 μl) of the lysate was used to perform the β-gal assay reaction by adding in 0.2 ml of 2mg/ml 2-nitrophenyl-β-D-galactopyranoside
substrate (ONPG from Roche). The variation of transfection efficiency was determined by OD_{420} reading on the intensity of β-gal activity of a co-transfected pRSVZ plasmid. The rest of the lysate was heated and prepared for loading on a thin layer chromatography (TLC) plate (Whatman). The loading amount used was adjusted based on the β-gal OD reading. The CAT reaction mixture was prepared by adding the proper amount of cell lysate, 30 μl of 4 mg/ml acetyl coenzyme A (Sigma), 0.2 μCi of C^{14}-labeled chloramphenicol (Amersham) and the proper amount of 0.25M Tris-HCl (pH=7.8) to a final volume of 150 μl. The mixtures were then incubated at 37°C overnight and extracted with 1ml of ethylacetate (Fisher). The ethylacetate extract was concentrated to 25 μl and spotted on a TLC plate. The TLC plates were exposed to X-OMAT film (Kodak) for up to two days.

**Western blot analysis of fusion protein**

Stable transfectants cultured in 90-mm dishes were washed with PBS twice and subjected to purification through His-Bind Quick 300 Cartridges (Novagen). Briefly, 1.0 ml ice-cold 1x binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH=7.9) with 0.1% NP-40 (Sigma) was added to each dish for lysis; then the lysates were collected and sonicated before applying to the cartridges. After eluting from the cartridges, samples were concentrated by spinning in Microcon-10 Microconcentrators (Amicon). The proteins were separated by 12% SDS-PAGE and transferred to the Immobilon-P membrane (Millipore). The membrane was blocked with 10% casein, incubated for 1 hr with primary antibody, Anti-Xpress (Invitrogen), followed by 1hr incubation with alkaline phosphatase conjugated secondary antibody, anti-mouse IgG (Novagen). NBT/BCIP (Boehringer Mannheim) was used as the substrate for detecting the signal.

**Immunocytochemistry**

Approximately 3-5 x10^4 cells were plated on a single-welled Lab-Tek II Chamber Slide (Nalge Nunc International) and cultured overnight. Cells were then washed with 37°C pre-warmed, serum-free medium three times, dehydrated, and fixed with 100% cold methanol for 5 minutes, then air dried. The dried cells were re-hydrated with 1X PBS for 10 minutes at room temperature, blocked with 3% BSA for 30 minutes, and then incubated with the primary antibody, Anti-Xpress (Invitrogen) for one hour. After an additional hour of incubation with secondary antibody, alkaline phosphatase conjugated anti-mouse IgG (Novagen), the NBT/BCIP (Roche) was used as the dye substrate. The slides were dehydrated with ethanol, cleared with Hemo-De (Fisher) and mounted with SuperMount (E.K. Industries, Inc.). Results were observed under an Olympus Research Microscope, Model AHBS (Olympus Optical Co. Ltd).

**RESULTS**

*Nkx-2.3 expression in the mouse epidermis*

To determine if Nkx-2.3 is expressed in the mouse epidermis, the relative amount of mouse Nkx-2.3 transcript in various tissues including epidermis was examined by a “same-tube RT-PCR” method. As shown in Figure 1, the Nkx-2.3 band was detected in the mouse epidermis that was separated from the dermis of the skin by treating with Dispase II (lane 2). The absence of the Nkx-2.3 band in liver (lane 5), a negative control tissue, and the presence of a good amount of the GAPDH band, an internal control, demonstrated the appropriate RNA preparation and the proper PCR condition used. The use of the “same-
tube RT-PCR” method eliminates the quantitative variation derived from the sample preparation in different tubes. Therefore, the result showed the strongest expression of \( Nkx-2.3 \) in the intestine (lane 4), and about the same amount of expression in the epidermis and the heart (lanes 2 and 3 respectively) as the loading control (GAPDH) was in similar amounts. Using an \( Nkx-2.3 \) gene-specific primer set, the presence of \( Nkx-2.3 \) transcript in the mouse epidermis was proven for the first time.

\( Nkx-2.3 \) expression in a mouse epidermal keratinocyte cell line

In \textit{Xenopus} skin, the \( XNkx-2.3 \) gene has been shown to be expressed in the epidermal keratinocytes (Ma, 2004). It would be also interesting to see if the \( Nkx-2.3 \) gene is expressed in the mouse epidermal keratinocytes. A mouse epidermal keratinocyte cell line (gift of Dr. Angela L. Tyner) was examined. It was used because of a concern about the contamination of other cell types in the epidermal tissue or the primary keratinocyte culture. Thus, the RT-PCR signal derived from keratinocytes would be indistinguishable from that of the contaminated cells. In addition, this line has been proven for its capability to respond to high-\( Ca^{2+} \) treatment, which then induces the terminal differentiation markers, such as loricrin, involucrine and transglutaminase (Vasioukhin and Tyner, 1997). Therefore, this keratinocyte cell line is mature and can lead to terminal epidermal differentiation. Figure 2 shows the keratinocyte cultures from both high-\( Ca^{2+} \) (lane 3) and low-\( Ca^{2+} \) concentrations (lanes 4 and 5) expressed \( Nkx-2.3 \) gene. This result implies that \( Nkx-2.3 \) transcript is indeed in the mouse epidermal keratinocytes. The specificity of the RT-PCR product of \( Nkx-2.3 \) gene was tested by cutting with \( Ava \) II and yielding the expected 91-bp and 53-bp bands (lane 5). A complete cut of the \( Nkx-2.3 \) band by \( Ava \) II meant the purity of this band. The density ratios of \( Nkx-2.3 \) band to \( \beta \)-actin band in keratinocytes were 2.0 and 1.1 for lanes 3 and 4 respectively. These ratios represented the relative abundance of the \( Nkx-2.3 \) transcript. Thus, the relative amount of \( Nkx-2.3 \) transcript was about 2-fold higher in

![Figure 1](image)

**Figure 1:** Expression of \( Nkx-2.3 \) in the mouse tissues
Using primers specific for the 3'-UTR of mouse \( Nkx-2.3 \), same-tube quantitative RT-PCR showed the expected 144 bp DNA band produced from epidermis, heart, and intestine, but not liver. GAPDH cDNA, the 407 bp band, served as an internal control.
cells induced to terminally differentiate with high-Ca\textsuperscript{2+} treatment (Figure 2, lane 3 labeled as “Ca\textsuperscript{2+}”) than in cells without high-Ca\textsuperscript{2+} treatment (lane 4 labeled as “no Ca\textsuperscript{2+}”). The possible role of \( Nkx-2.3 \) in the Ca\textsuperscript{2+}-induced terminal differentiation of keratinocytes needs further investigation. Nonetheless, the expression of the \( Nkx-2.3 \) gene in the mouse epidermal keratinocytes was confirmed.

**Sequence similarity between \( XNkx-2.3 \) and mouse \( Nkx-2.3 \)**

The temporal and spatial expression patterns and the T\textsubscript{3} regulation of \( XNkx-2.3 \) gene make it fit the role in the maturation of the frog epidermis (Ma, 2004). Although TH is not the epidermal maturation trigger in the mouse, the mouse and *Xenopus* \( Nkx-2.3 \) proteins are about 58% identical in their amino acid sequences, and all of their four functional domains, TN (tinman), homeodomain, NK-2 SD, and GIRAW, are well preserved except for one amino acid in the homeodomain (Figure 3). This implies that the function of \( Nkx-2.3 \) in cells may likely be conserved between these two species.

**Spontaneous aggregates found in \( XNkx-2.3 \)-transfected pluripotent P19 cells**

To test if the \( Nkx-2.3 \) protein has the conserved function between frog and mouse, the *Xenopus* \( Nkx-2.3 \) gene was transfected into a mouse pluripotent embryonic carcinoma (EC) cell line, P19, to determine if over-expressing \( XNkx-2.3 \) would channel a pluripotent cell line towards the adult epidermal fate.

![Figure 2: Expression of \( Nkx-2.3 \) in a mouse keratinocyte cell line](image)

The result of same-tube quantitative RT-PCR showed the expected 144 bp DNA band produced from RNA of both Ca\textsuperscript{2+} (lane 3) and non-Ca\textsuperscript{2+}-treated (lane 4) keratinocytes. An internal cut of restriction enzyme (Ava II) was used to confirm the specificity and the purity of \( Nkx-2.3 \) cDNA fragment. The expected 91 bp and 53 bp bands were yielded after Ava II cutting (lane 5). A shorter gel running time gave a clear 53 bp band in lane 7. Mouse \( \beta \)-actin, the 279 bp cDNA fragment, was the internal control. The 1 Kb DNA ladder (Gibco-BRL) was used as a molecular weight marker. The first band on the top of lane 1 is 506 and 517 bp fragments.
Introducing the *Xenopus* gene into the mouse cell line was due to the lack of appropriate *Xenopus* pluripotent cell line. The mouse P19 cell line was chosen because its clonal sublines are readily obtained at high frequency and the transfected clones retain their ability to differentiate into the cell types derived from all three embryonic germ layers (Bain et al., 1994; McBurney and Rogers, 1982; Rossant and McBurney, 1982). Several Zeocin-resistant cell clones were selected. Taking advantage of the His tag in pCDNA4/HisMax mammalian expression system, the cell extract of the clone was partially purified through a Ni-column. Using Western blot of the Ni-purified protein with antibody against Xpress (an epitope tag in the pCDNA4/HisMax), 2 stable transfectants clones 6 and 7, were identified to express *XNkx-2.3*-Xpress fusion protein of the expected size (the two fine bands indicated by an arrow in Figure 4A, only clones 1 to 7 shown). The expression of the *XNkx-2.3*-Xpress fusion protein in clones 6 and 7 was further confirmed by immunocytochemical staining of all Zeocin-selected clones with antibody against Xpress (Figure 4B, only clones 2, 3, 6, and 7 shown). During routine culturing, clone 6 and clone 7 tended to aggregate spontaneously (indicated by arrowheads in Figures 4B and 5A), which is a differentiation phenomenon of embryonic carcinoma cell (Martin and Evans, 1975).

**Induction of K1 in XNkx-2.3-P19 stable transfectants**

In order to investigate any epidermal differentiation occurring among *XNkx-2.3* transfectants, the “same-tube RT-PCR” was used to examine the expression of K8, K5, and K1 keratins. Keratins are excellent

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**Figure 3:** Sequence comparison of the Nkx-2.3 proteins between mouse and *Xenopus*. Four functional domains, TN, homeodomain, NK-2, and GIRAW, are labeled as indicated in the box. “+” represents a high similarity of the amino acid in the position. The only amino acid difference (asterisk) among all four functional domains is in homeodomain. The sequence comparison was performed on “NCBI-Blast 2 Sequences” using BLOSUM62 matrix.
markers for epidermal development. As mentioned previously, the epidermal ontogenesis has been well defined in the mouse by examining the expression pattern of keratin markers. Among the four cell clones tested, clones 5-8, the K8 or K5 transcripts could not be detected by RT-PCR using several sets of primers (data not shown). However, the adult keratin, K1 (the lower bands in Figure 5), was found in clone 6 and clone 7, which were the two clones expressing XNkx-2.3-Xpress fusion protein, but not in clone 5, clone 8, or non-transfected P19 cells (Figure 5B). This result suggests that the adult K1 keratin could be induced by the over-expression of Nkx-2.3 gene in P19 pluripotent cells.

The Xenopus Nkx-2.3 gene can not directly activate frog 63KDa keratin promoter

To test if the XNkx-2.3 gene can directly activate the adult keratin, the CAT assay was performed to study the activator-reporter relation between the XNkx-2.3 gene and the 63 KDa keratin 5’ regions of various lengths in several mammalian cell lines, including Hela, NIH3T3, HaCaT,

Figure 4: Stable XNkx-2.3-P19 clones expressed the XNkx-2.3-Xpress fusion protein. (A) 2 out of the 7 drug-resistant clones, 6 and 7, were positive for expression of XNkx-2.3-Xpress fusion protein detected by the Western blot using Anti-Xpress antibody. The molecular size of the fusion protein was about 40 KDa, as indicated by arrow. A non-specific unknown band existing in every partial purified sample was used as the loading control. (B) Immunostaining of the fusion protein with Anti-Xpress confirmed that clones 6 and 7 had the strongest expression. The signals for clones 2 and 3, which did not have the 40 KDa band in the Western blot, were not stained differently from the untransfected P19 cells. Arrowheads indicate the aggregates in the cultures. Scale bar =100μm
P19, and the XNkx-2.3-P19 stable transfectants. There are several potential Nkx-2.3 protein binding sites in both the 63 KDa and K1 keratin gene promoters. In fact, a perfect DNA consensus core (5’-CAAGTG-3’) of the NK-2 homeodomain binding site was found at -366 to -361 bp of the 63 KDa gene according to the known sequence (Weiler et al., 1998). In the case of using P19 as the host, cells were transfected using the 63 KDa keratin gene promoter-reporter constructs, pCAT-5200, pCAT-2300, or pCAT-630 (Figure 6A) with or without the presence of XNkx-2.3 plasmid, pCS2+-XNkx2.3. Comparing with the RSV-CAT activity, no activation effect was observed on any one of the three keratin promoter-reporter constructs in P19 cells. Figure 6B shows the results of using 5 μg of expression plasmid. The Tris buffer was used as a background control; the CAT-basic was a negative control CAT activity without a promoter and the RSV-CAT is a positive control with RSV promoter. In fact, the input of 20 μg of XNkx-2.3 expression plasmid had no activation effect on all three promoter-reporter constructs (Figure 6, C showed the pCAT-630 result only). Although the slightly weaker CAT signals were observed when adding 5 μg of expression plasmid, this weak inhibition was only found in P19 cells but not in other host cell lines used (data not shown) or other DNA amounts (10 μg or 20 μg) used (Figure 6C). Another noticeable difference in P19 cells transfection was that the CAT-Basic construct gave high background on the basic CAT activity, which was not seen in other cell lines. Overall, the results of the CAT assay indicated that Nkx-2.3 gene could not directly activate the 63 KDa keratin promoter in several mammalian host cells that were tested.

Figure 5: Spontaneous aggregation and K1 induction in the XNkx-2.3-P19 stable clones. (A) An XNkx-2.3-P19 stable transfectant, clone 6, showed spontaneous formation of aggregates (indicated by arrowhead) in a normal culture condition. In contrast, the same density of control P19 cells grew in monolayer without aggregation. Scale bar =100μm (B) Mouse K1 keratin (mK1), a 216-bp fragment, was found to be expressed in clones 6 and 7 detected by the “same-tube RT-PCR”. Clones 5 and 8 identified as weaker expression clones by Anti-Xpress staining did not have K1 induction. The β-actin was used as an internal control.
DISCUSSION

The present study demonstrated for the first time that the expression of *Nkx-2.3* gene is in the mouse epidermis and the mature keratinocytes. The Nkx-2.3 proteins of mouse and *Xenopus* are almost identical for all four functional domains. In this study, the cross-species induction of the adult K1 keratin in a mouse pluripotent cell line by the *Xenopus Nkx-2.3* gene implies that this gene may have a conserved role in epidermal maturation for both species. However, the mechanism of the induction is still unknown.

As shown in Figures 1 and 2, mouse *Nkx-2.3* was indeed expressed in both the adult mouse epidermis and the mature

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**Figure 6:** CAT assay for co-transfection of the *XNkx-2.3* gene and the 63 KDa keratin promoter.  
(A) The 5.2 kb 5' region of the 63 KDa keratin gene was digested with different restriction enzymes to produce the 2.3 kb and 630 bp fragments for constructing the CAT reporter system (Materials and Methods). (B) The 63 KDa keratin promoter constructs (5200-CAT, 2300-CAT, 630-CAT) were transfected into P19 cells with (+) or without (-) the *XNkx-2.3* gene. The number before the CAT presented for the length (in base pair) of the promoter. The Tris buffer, CAT-basic, and RSV-CAT were used as the background, negative and positive controls respectively. (C) Various amounts of the pCS2+-XNkx2.3 plasmid were tested using 630-CAT reporter construct. The cell extract for CAT reaction were adjusted by OD_{420} readings of the total proteins and the β-gal activity from the expression of a co-transfected lacZ plasmid, pRSVZ. The pCS2+ and the pBluescripts-KS vectors were added to even the quantity difference of pCS2+-XNkx2.3 and pCAT-promoters used in P19 cells respectively.
epidermal keratinocyte cell line, which are adult differentiation program ready. According to the expression patterns of *Xenopus Nkx-2.3* in the developing epidermis of tadpoles, this gene is considered as an important marker for epidermal maturation (Ma, 2004). In addition, as described in the introduction, several features of skin maturation are well conserved between the frog and the mouse. Together, the presence of a good amount of *Nkx-2.3* transcript in mature mouse epidermal keratinocytes implies that the *Nkx-2.3* gene may play a conserved role in skin maturation of the two organisms.

In *Xenopus*, the heart also expresses *XNkx-2.3* gene (Evans et al., 1995; Ma, 2004). The results in this study show that the mouse heart has a significant amount of *Nkx-2.3* transcript (Figure 1, lane 3; Figure 2, lane 2), which is in contrast to a previous study using RNase protection assay (Biben et al., 2002). The reason for this difference is not clear. However, the specificity of the PCR product in this study was carefully examined by sequencing and by checking an internal *Ava* II cut (lane 5 in Figure 2). In addition, the use of DNase-treated RNA excluded the possibility of genomic DNA contamination in the RT-PCR template. The absence of DNA contamination in the RNA sample of this study was also evidenced by the absence of a 500 bp intron-containing DNA fragment that would be amplified by the β-actin primers in the RT-PCR result (Figure 2). Hence, the detection of *Nkx-2.3* product in the heart sample is unlikely the noise signal from PCR amplification or a result from genomic DNA contamination.

The observation of *XNkx-2.3* transected P19 cells exhibiting spontaneous aggregation in the normal culture condition suggests that these cells might have changed their fate. The aggregation through cell suspension in the differentiation cultures of ES or EC cells is known to be mediated by E-cadherin (Dang et al., 2004), which also plays an important role in epidermal terminal differentiation in vivo (Dang et al., 2004; Tinkle et al., 2004). Using R1 ES cells and a special two-step culture process including the suspension method to form cell aggregates, Troy and Turksen were able to produce almost 100% of K14-positive early EPC (epidermal progenitor cells). From the EPC, they can simulate the sequential differentiation events of the epidermal cell fate, which is similar with those occurring during the normal mouse development, although they only generated the K1-expressing cells in a low efficiency manner when using the medium in which the Ca$^{2+}$ concentration (1.8 mM) is high enough to induce the terminal differentiation (Troy and Turksen, 2005; Turksen and Troy, 1998). Taken together, aggregation is an important step for epidermal differentiation. Although the relationship between the E-cadherin and the differentiation needs to be further studied, the aggregation phenomenon was observed and related to the over-expression of *Xenopus Nkx-2.3* protein in the mouse pluripotent P19 EC cell line.

The possibility of conserved role of *Nkx-2.3* gene in epidermal maturation was further shown by the K1 keratin induction in the mouse P19 cells transfected with the *Xenopus Nkx-2.3* gene. In previous epidermal differentiation study on ES cells, K1-expressing cells only appeared in the culture regions of very high cell density (Troy and Turksen, 2005), where ES cells may differentiate into all types of tissues randomly. The K14-positive EPC derived from ES cells is less mature than the K14-expressing basal cells isolated from newborn skin (Troy and Turksen, 2005). The newborn basal cells easily undergo terminal differentiation when the Ca$^{2+}$ concentration is elevated to 1.2 mM (Hennings et al., 1980), while for EPC it is more difficult. One of the possibilities is due to the lack of maturation. This observation is consistent with the developmental process of mouse epidermis, in which the K14-expressing basal cells appear at E9.5, but do not terminally differentiate until the maturation program is ready by E15.5 (Byrne et al., 1994). Although the percentage of K1-expressing cells in the stable transfectants was not determined, it showed that K1 transcript was detectable in a significant amount when the *XNkx-2.3* was over-expressed in a pluripotent embryonic P19 cell line without
increasing the cell density, raising the calcium concentration, or performing suspension method in the culture. In addition, the two K1-positive XNkx-2.3-P19 transfectants, clones 6 and 7, did not show cornification or up-regulation of terminal differentiation markers, such as involucrin and transglutaminase (data not shown) in this study. This means that the stable clones were not in the status of terminal differentiation, even though the K1 was present. Thus, the induction of K1 gene by over-expressing XNkx-2.3 may not be a result of the terminal differentiation that happens only after maturation.

Unexpectedly, the CAT assay using the transient transfection on the Xenopus equivalent of mammalian K1 in P19 cells did not reflect that this K1-induction was a direct activation. These repugnant results from stable and transient transfection experiments may result from the sequence difference of the promoters between mouse K1 and Xenopus 63 KDa keratins. However, this possibility can be excluded, because the regulatory mechanisms of the promoters between 63 KDa keratin gene and K1 keratin gene are known to be well conserved on both the basic transcriptional factors and several cis-elements (Warshawsky and Miller, 1995). Another possibility could be that the factors required to activate the keratin gene are simply not pre-equipped in the cells. The factors that act in concert with Nkx-2.3 protein to induce mouse K1 in the stable XNkx-2.3-P19 clones may gradually appear from a process initiated by the stably expressed XNkx-2.3 protein. The pluripotent cells, therefore, may become different from the XNkx-2.3 stable clones and the transient transfected cells. From the observation of spontaneous aggregation occurring only in the XNkx-2.3-P19 stably transfected clones, it was likely that the transfected cells began their differentiation process in a normal culture condition, even though the pluripotent cells were still growing. The interaction between cells in the aggregates and the presence of the over-expressed Nkx-2.3 protein in the transfected cells may lead some cells into the mature epidermal fate. However, this hypothesis needs to be confirmed by further elaborating the detailed expression patterns of several ventral ectoderm factors and epidermal lineage markers in the aggregates.

Although expression of Nkx-2.3 transcript in adult keratinocytes and induction of adult keratin by Nkx-2.3 gene in pluripotent cells were observed in this study, a role of Nkx-2.3 gene during the skin maturation process remains to be uncovered. Nkx-2.3 is a member of NK-2 homeodomain protein family which may be regulated by BMPs (BMP-2 and 4 especially) and Wnt signals (Harvey, 1996). They were all critical for epidermal fate (Botchkarev and Sharov, 2004; Saitoh et al., 1998). It would be interesting to see if these signals change during skin maturation and cause a change of Nkx-2.3 expression level. However, more complexity of these signal interactions with Nkx-2.3 gene should be noted because down-regulation of the BMP-2 and BMP-4 caused by disruption of Nkx-2.3 gene was observed and suggested in the mouse intestine (Pabst et al., 1999).

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