Table 1
Distribution of genotype and allele frequencies for *G6PD* polymorphisms in Gujarat vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n = 449)	Patients (n = 366)	*p* value[a] for Association	OR	95% CI
3'UTR A/G (rs1050757)	AA	239 (0.53)	142 (0.39)	R	1	
	AG	170 (0.38)	181 (0.49)	<0.0001	1.792	1.334–2.407
	GG	40 (0.09)	43 (0.12)	0.014	1.809	1.122–2.919
	A	648 (0.72)	465 (0.64)	R	1	
	G	250 (0.28)	267 (0.36)	0.0002	1.488	1.207–1.836
Exon 6 C/T (Ser218Phe; rs5030868)	CC	444 (0.99)	361 (0.986)	R	1	
	CT	3 (0.006)	4 (0.011)	0.515	1.640	0.364–7.377
	TT	2 (0.004)	1 (0.003)	0.689	0.615	0.055–6.813
	C	891 (0.99)	726 (0.99)	R	1	
	T	7 (0.01)	6 (0.01)	0.928	1.052	0.351–3.145
Exon 11 C/T (Tyr467Tyr; rs2230037)	CC	446 (0.993)	363 (0.992)	R	1	
	CT	3 (0.007)	3 (0.008)	0.801	1.229	0.246–6.126
	TT	0 (0.0)	0 (0.0)	–	–	–
	C	895 (0.99)	729 (0.99)	R	1	
	T	3 (0.003)	3 (0.004)	0.802	1.228	0.247–6.103

n: number of subjects; R: reference group; OR: Odds Ratio; CI: Confidence Interval.
[a] Vitiligo Patients *vs.* Controls using chi-squared test with 2 × 2 contingency table.

compared to normal melanocytes. Therefore, vitiligo may result from an insufficient response to oxidative stress and impaired G6PD levels.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

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References

[1] N.C. Laddha, M. Dwivedi, M.S. Mansuri, et al., Vitiligo: the interplay between oxidative stress and immune system, Exp. Dermatol. 22 (2013) 245–250.
[2] A. Farahi-Jahromy, M.K. Fallahzadeh, S. Ashkani-Esfahani, et al., Decreased glucose-6-phosphate dehydrogenase levels in vitiligo patients: further evidence of oxidative stress, Adv. Biomed. Res. 3 (2014) 34.
[3] M.S. Mansuri, M. Singh, R. Begum, miRNA signatures and transcriptional regulation of their target genes, J. Dermatol. Sc. 84 (2016) 50–58.
[4] F. Amini, E. Ismail, 3'-UTR variations and G6PD deficiency, J. Hum. Genet. 58 (2013) 189–194.
[5] N.C. Laddha, M. Dwivedi, M.S. Mansuri, et al., Role of oxidative stress and autoimmunity in onset and progression of vitiligo, Exp. Dermatol. 23 (2014) 345–368.
[6] M.S. Mansuri, N.C. Laddha, M. Dwivedi, et al., Genetic variations (Arg5Pro and Leu6Pro) modulate the structure and activity of GPX1 and genetic risk for vitiligo, Exp. Dermatol. 25 (2016) 654–657.

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Letter to the Editor

Simple cell culture media expansion of primary mouse keratinocytes

Letter to the editor

Keratinocytes are the majority cell population in the epidermis and protect the body from the environment by forming stratified layers with successively differentiated keratinocytes. The study of keratinocyte biology has yielded many important insights, both through *in vivo* mouse models, and *in vitro* culture systems. Given the highly dynamic genetic systems available for *in vivo* mouse investigation, it is important to culture primary mouse keratinocytes derived from these models in isolation for further mechanistic study *in vitro*. Despite the need, culturing mouse primary keratinocytes has been difficult.

With existing protocols for the isolation and culture of mouse keratinocytes (MKC) [1–3], cells often do not passage consistently, and often lose features of undifferentiated basal layer cells. Moreover, many protocols in the literature are inconvenient because of either the use of feeder cells, or the use of a large repertoire of individual distinct supplements such as growth factors. Previously, Chapman et al. found that Y-27632, a Rho kinase inhibitor, robustly enhances proliferative capacity and maintains original characteristics of human keratinocytes [4]. However, the effect of Y-27632 on culturing primary mouse keratinocytes has not been reported. Given the deficiencies in current common techniques, our goal was to test simple cell culture media preparations using the Y-27632 to streamline primary cultures of mouse keratinocytes.

Rho kinases (ROCKs) are the first downstream mediator of the GTP-binding protein RhoA and have pleiotropic functions including the regulation of cellular contraction, migration, morphology, polarity, and cell division [5]. ROCKs exert their functions by phosphorylating different substrates such as myosin light chain (MLC), LIM kinase (LIMK), and phosphatase and tensin homologue

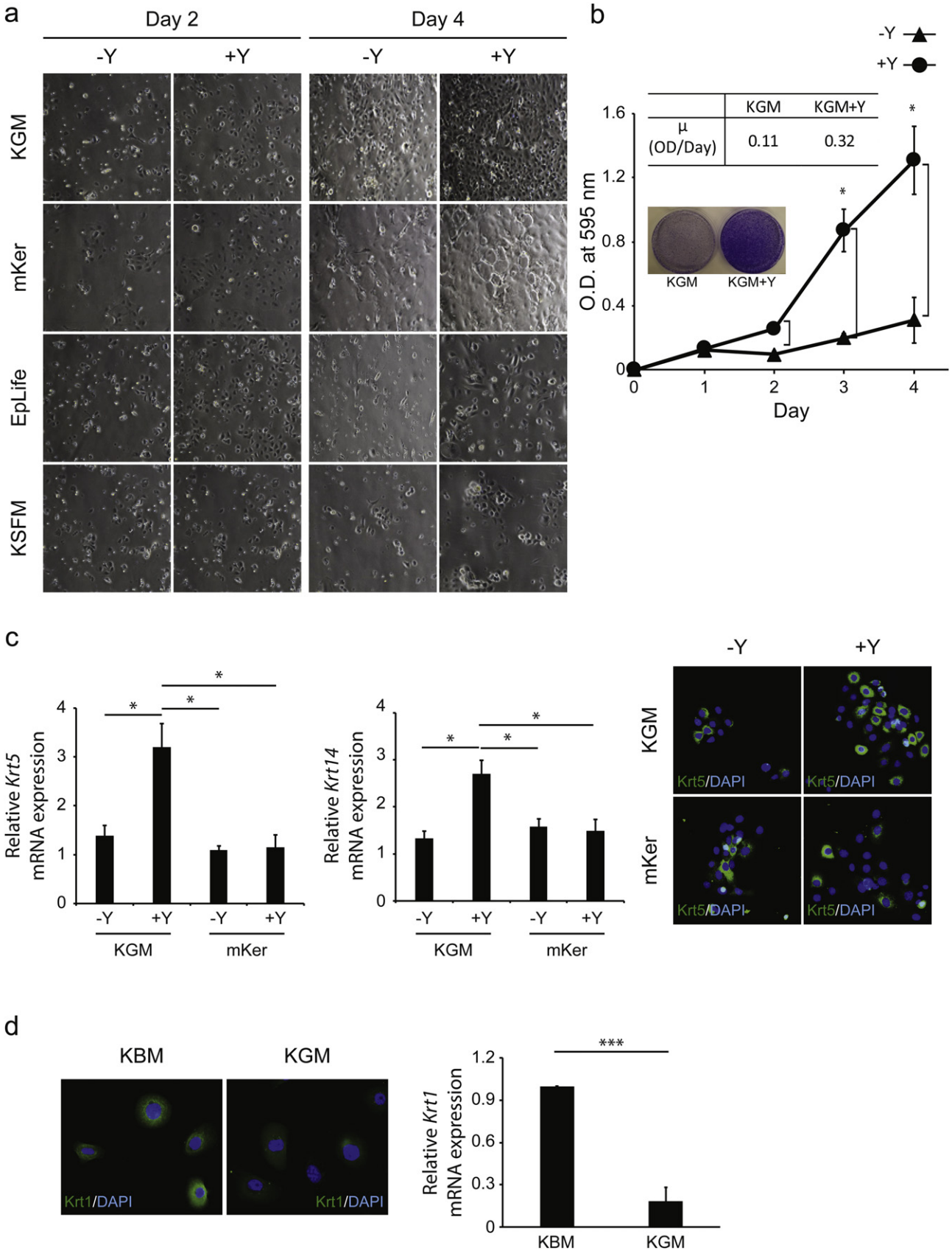

Fig. 1. ROCK inhibitor enhances MKC proliferation. (a) The cell morphology of mouse keratinocytes cultured in different media with or without Y-27632 (10 μM). (b) Proliferation assay of keratinocytes cultured in KGM media using crystal violet. (c) mRNA expression and immunofluorescence staining of Krt5 and Krt14 for comparing KGM and mKER medias with or without Y-27632. (d) mRNA expression and immunofluorescence staining of Krt1 to test the capacity of cell differentiation after expansion in KGM with Y-27632. $^{*}P < 0.05$, $^{***}P < 0.001$.

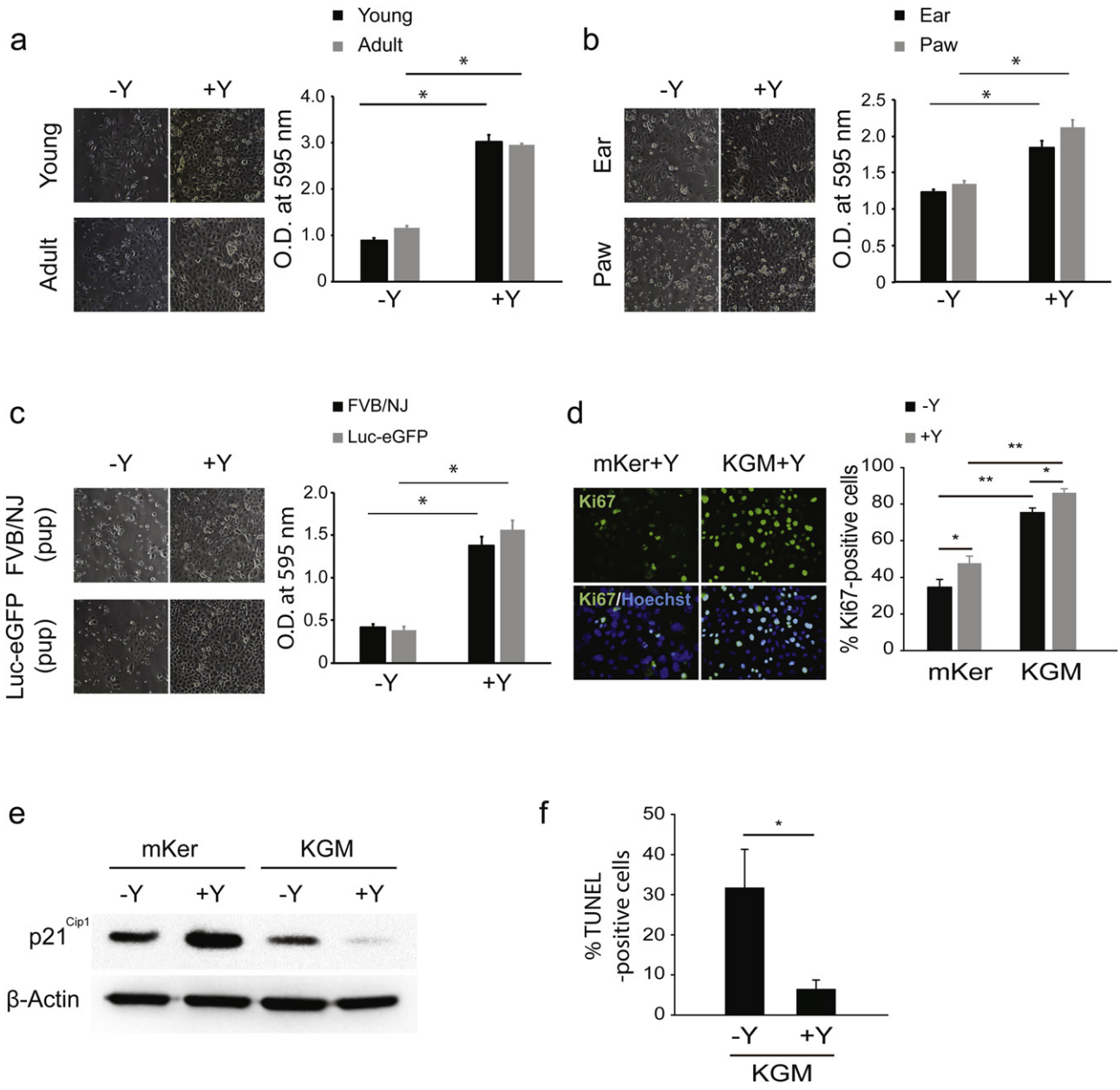

Fig. 2. ROCK inhibitor enhances proliferation of MKC regardless of mouse age, skin type, and strain. The morphology and proliferation assay of keratinocytes isolated from (a) young and aged C57BL/6 J mice, (b) different skin sites (*i.e.*, paw and ear) of young C57BL/6 J mice, and (c) pups of FVB/NJ and Luc-eGFP transgenic mice cultured in KGM with or without Y-27632. (d) Immunofluorescence staining of Ki67+ and quantification of %Ki67+-positive cells after expansion in KGM or mKER with or without Y-27632. (e) Western blotting of $p21^{Cip1}$ detected from mouse keratinocytes cultured in KGM or mKER with or without Y-27632. (f) Quantification of %TUNEL-positive cells cultured in KGM with or without Y-27632. $^{*}P < 0.05$, $^{**}P < 0.01$.

(PTEN), which are involved in stress fiber assembly, contraction, actin stabilization through cofilin inhibition, actin-membrane linkage, intermediate filament disruption, and proliferation [6]. Also, the ROCK inhibitors have been widely used to enhance the cloning and survival efficiency of human embryonic stem cell [7,8]. Here, we describe the simple and convenient method for primary culture of mouse keratinocytes by adding Y-27632 to a subset of commercially and commonly available base keratinocyte media. We assessed the success of ROCK inhibition on MKC primary cultures derived from mice of different ages, body sites and strains.

MKC were isolated from the skin of neonatal pups and tails of adult mice by following the protocol reported previously [1]. We randomly picked three pre-formulated commercially available media for human keratinocytes and the common recipe-based media for MKC (mKER) (Supplement Table S1) [9]. Freshly isolated MKC from pups of C57BL/6 J mice were cultured in each media with 10 μM of Y-27632 (Fig. S1a) and changed with media every 2 days until cells are confluent. We initially tested all media by evaluating the cellular morphology. On day 2 after isolation, although cells are morphologically identical in all media, we observed more cells attached in every media supplemented with Y-27632 (Fig. 1a). However, on day 4, cells cultured in KGM (Keratinocyte Growth Media) specifically grow better than those in other media. Moreover, cell density was increased even more in KGM supplemented with Y-27632 (Fig. 1a and b). In contrast– although mKER is perhaps the most frequently used for culturing mouse keratinocytes– mKER resulted in the lowest cell proliferation and the highest cell differentiation during culturing among the four media tested, regardless of the use of Y-27632. These results indicate that ROCK inhibition maintains a homogenous population

and enhances cell proliferation of primary MKC, particularly those cultured in KGM.

To determine whether ROCK inhibition maintains basal layer qualities of MKC, the levels of Keratin (Krt)5 and Krt14 were evaluated by RT-qPCR and immunofluorescence. We found that MKC cultured in KGM containing Y-27632 express more Krt5 and Krt14 than cells in KGM alone and certainly mKER with or without Y-27632 (Fig. 1c). Next, as a functional study, we tested the differentiation ability of MKC cultured in KGM with Y-27632 by evaluating differentiation markers such as *Krt1*, *Krt10*, *Filaggrin* (*Flg*) and *Loricrin* (*Lor*). For this experiment, after expansion in KGM with Y-27632, cells were incubated in KBM without growth factors to induce differentiation [10]. All differentiation factors were significantly increased (Figs.1d and S1b). In particular, immunostaining experiment confirmed Krt1 expression in MKC cultured in KBM (Fig. 1d). These results indicate that MKC expanded in KGM with Y-27632 maintain a basal keratinocyte phenotype, but are still capable of differentiation upon growth factor withdrawal.

We next test whether Y-27632 supports keratinocytes culture regardless of mouse age, skin type, and strain. MKC were isolated from tails of young (~3 weeks old) and adult mouse (more than 2-month old), paw and ear skin, and neonatal mice of FVB/NJ and Luciferase-eGFP (Luc-eGFP) transgenic mice. Similar to Fig. 1, KGM supplemented with Y-27632 successfully enhanced proliferation of MKC independent on age, skin type, and strain (Fig. 2a–c).

Given that Y-27632 enhances proliferation of MKC, we examined the expression of $Ki67^{+}$ and $p21^{Cip1}$ as a positive and negative features of cell proliferation, respectively. We observed that KGM condition supports more Ki67 positive cells than the mKER and Y-27632 slightly increases the frequency of Ki67 positive cells (Fig. 2d). Additionally, $p21^{Cip1}$ expression is significantly decreased in MKC cultured in KGM with or without Y-27632, compared to MKC cultured in mKER with or without Y-27632 (Fig. 2e). Lastly, Terminal dUTP nick end labeling (TUNEL) assay confirmed that Y-27632 decreases the number of apoptotic cells (Fig. 2f). These results indicate that ROCK inhibition promotes proliferation and maintains cell survival by regulating cell-cycle dependent mechanisms.

In conclusion, using this simple method with addition of Y-27632 (especially to KGM media), we consistently grow epidermal keratinocytes from mouse skin independent on age, skin type, and strain including transgenic mice expressing luciferase and GFP. This represents a significant improvement from the common mKER media that precludes extensive testing of primary mouse keratinocytes. Like most primary cells, MKC cultured even in our ideal conditions still did not divide beyond passage 3 (Fig. S1e), indicating that more work remains to be done. However, up to this point the MKCs can be expanded and differentiated in this novel *in vitro* protocol. Moreover, MKCs can be cryopreserved for the long-term storage and the survival rate is about more than 90% after thawing (Fig. S1f). Therefore, this method should improve use of mouse models for *in vitro* study of epidermal biology.

Conflict of interest

The authors declared that no conflict of interest exists.

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Appendix A. Supplementary data

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References

[1] U. Lichti, J. Anders, S.H. Yuspa, Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice, Nat. Protoc. 3 (5) (2008) 799–810.
[2] L. Li, Mouse epidermal keratinocyte culture, Methods Mol. Biol. 945 (2013) 177–191.
[3] F. Li, C.A. Adase, L.-j. Zhang, Isolation and culture of primary mouse keratinocytes from neonatal and adult mouse skin, J. Vis. Exp. (125) (2017) , doi:http://dx.doi.org/10.3791/56027.
[4] S. Chapman, X. Liu, C. Meyers, R. Schlegel, A.A. McBride, Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor, J. Clin. Invest. 120 (7) (2010) 2619–2626.
[5] K. Riento, A.J. Ridley, ROCKs: multifunctional kinases in cell behaviour, Nat. Rev. Mol. Cell Biol. 4 (2003) 446.
[6] E. Amin, B.N. Dubey, S.C. Zhang, L. Gremer, R. Dvorsky, J.M. Moll, M.S. Taha, L. Nagel-Steger, R.P. Piekorz, A.V. Somlyo, M.R. Ahmadian, Rho-kinase: regulation, (dys) function, and inhibition, Biol. Chem. 394 (11) (2013) 1399–1410.
[7] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J.B. Takahashi, S. Nishikawa, S. Nishikawa, K. Muguruma, Y. Sasai, A ROCK inhibitor permits survival of dissociated human embryonic stem cells, Nat. Biotechnol. 25 (6) (2007) 681–686.
[8] G. Chen, Z. Hou, D. Gulbranson, J.A. Thomson, Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells, Cell Stem Cell 7 (2) (2010) 240–248.
[9] J. Reichelt, I. Haase, Establishment of spontaneously immortalized keratinocyte lines from wild-type and mutant mice, in: K. Turksen (Ed.), Epidermal Cells: Methods and Protocols, Second edition, Humana Press Inc., Totowa, 2010, pp. 59–69.
[10] D. Kim, M.Z. Hossain, A. Nieves, L. Gu, T.S. Ratliff, S. Mi Oh, A. Park, S. Han, N.B. Yang, J. Qi, J.M. Taube, S. Kang, L.A. Garza, To control site-specific skin gene expression, autocrine mimics paracrine canonical Wnt signaling and is activated ectopically in skin disease, Am. J. Pathol. 186 (5) (2016) 1140–1150.

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