and mesoderm in a manner similar with those of the 38C2 line. After day 5, however, 20D17 iPS cells did not differentiate into cardiomyocytes. The Oct3/4 level was much higher in 20D17 than 38C2 on days 8 and 10. It has been reported that the over-expression of Oct3/4 triggers the differentiation of cells into primitive endoderm and mesoderm,[22] and that elevated Oct4 at early stages (by day 2) is required for cardiomyogenesis.[23] In contrast, sustained over-expression of Oct3/4 in neural progenitor cells or myoblasts prevents their terminal differentiation.[24,25] The higher Oct3/4 levels at late stages might be attributable to the low efficiency of cardiomyocyte differentiation in 20D17.

A member of class II HDAC, HDAC4, binds to the transcription factors SRF and Mef2C and inhibits the expression of cardiac-specific genes.[16,17] The present study demonstrated that the nuclear protein level of HDAC4 was the highest in 20D17 and the lowest in 256H18 among all iPS cell lines. This difference in the nuclear HDAC4 levels might be involved, in part, in the variation of cardiomyocyte differentiation efficiency in different lines of iPS cells. In addition, by DNA microarray analysis, we showed that expressions of genes involved in cardiogenesis such as Wnt11, FGF4, and BMP4 are up- or down-regulated in 20D17. Further studies are needed regarding precise mechanisms by which these genes modulate cardiomyocyte differentiation in distinct iPS cell lines.

Narazaki *et al.* used iPS cell lines similar to those we employed but did not point out the variation of myocardial differentiation in different iPS cell lines.[5] They selected Flk-1-positive iPS cells and seeded these cells on dishes coated with collagen IV or OP9 feeder cells.[5] As we applied a simple system, myocardial differentiation by our protocol may be more susceptible to differences in gene or protein expression among distinct iPS cell lines.

In the clinical setting, cell line-to-line variation may occur when generating original iPS cells derived from patients with heart diseases. The forced acetylation of histones and transcription factors by HDAC inhibitors in patient-oriented iPS cells would be useful to overcome such variation and efficiently develop cardiomyocytes for the purpose of cell transplantation or drug screening.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgement

We thank M. Kiriyama for their excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported in part by the Project for Realization of Regenerative Medicine in the Life Science Program (T.N.), by a grant from the Foundation for Promotion of Research on Adult Vascular Diseases(K.H.), and by a research grant on Publicly Essential Drugs and Medical Devices from The Japan Health Sciences Foundation(K.H.).

References

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–676.
2. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;**448**:313–317.
3. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T *et al.* Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat biotechnol* 2008;**26**:101–106.
4. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**: 861–872.
5. Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S *et al.* Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* 2008;**118**:498–506.
6. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS *et al.* Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* 2008;**118**:507–517.
7. Schenke-Layland K, Rhodes KE, Angelis E, Butylkova Y, Heydarkhan-Hagvall S, Gekas C *et al.* Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. *Stem Cell* 2008;**26**:1537–1546.
8. Iida M, Heike T, Yoshimoto M, Baba S, Doi H, Nakahata T. Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. *FASEB J* 2005;**19**:371–378.
9. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T *et al.* Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* 2000; **408**:92–96.
10. Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 1997;**11**: 1061–1072.
11. Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 1993;**119**:419–431.
12. Kasahara H, Bartunkova S, Schinke M, Tanaka M, Izumo S. Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ Res* 1998;**82**:936–946.
13. Kawamura T, Ono K, Morimoto T, Wada H, Hirai M, Hidaka K *et al.* Acetylation of GATA-4 is involved in the differentiation of embryonic stem cells into cardiac myocytes. *J Biol Chem* 2005;**20**:19682–19688.
14. Yanazume T, Hasegawa K, Morimoto T, Kawamura T, Wada H, Matsumori A *et al.* Cardiac p300 is involved in myocyte growth with decompensated heart failure. *Mol Cell Biol* 2003;**23**:3593–3606.
15. Baba S, Heike T, Yoshimoto M, Umeda K, Doi H, Iwasa T *et al.* Flk 1(+) cardiac stem/progenitor cells derived from embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model. *Cardiovasc Res* 2007;**76**:119–131.
16. Davis FJ, Gupta M, Camoretti-Mercado B, Schwartz RJ, Gupta MP. Calcium/calmodulin-dependent protein kinase activates serum response factor transcription activity by its dissociation from histone deacetylase, HDAC4. Implications in cardiac muscle gene regulation during hypertrophy. *J Biol Chem* 2003;**278**:20047–20058.
17. Karamboulas C, Swedani A, Ward C, Al-Madhoun AS, Wilton S, Boisvenue S *et al.* HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage. *J Cell Sci* 2006;**119**:4305–4314.
18. Pandur P, Läsche M, Eisenberg LM, Kühl M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* 2002;**418**:636–641.
19. Barron M, Gao M, Lough J. Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. *Dev Dyn* 2000;**218**:383–393.
20. Olson EN, Schneider MD. Sizing up the heart: development redux in disease. *Genes Dev* 2003;**17**:1937–1956.
21. Yuasa S, Itabashi Y, Koshimizu U, Tanaka T, Sugimura K, Kinoshita M *et al.* Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells. *Nat Biotechnol* 2005;**23**:607–611.
22. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000;**24**: 372–376.
23. Zeineddine D, Papadimou E, Chebli K, Gineste M, Liu J, Grey C *et al.* Oct-3/4 dose dependently regulates specification of embryonic stem cells toward a cardiac lineage and early heart development. *Dev Cell* 2006; **11**:535–546.
24. Lang KC, Lin IH, Teng HF, Huang YC, Li CL, Tang KT *et al.* Simultaneous overexpression of Oct4 and Nanog abrogates terminal myogenesis. *Am J Physiol Cell Physiol* 2009; **297**:C43–C54.
25. Okuda T, Tagawa K, Qi ML, Hoshio M, Ueda H, Kawano H *et al.* Oct-3/4 repression accelerates differentiation of neural progenitor cells in vitro and in vivo. *Brain Res Mol Brain Res* 2004;**132**:18–30.

Allergology International. 2010;59:105-113
DOI: 10.2332/allergolint.09-RAI-0160

REVIEW ARTICLE

The Inflammasome, an Innate Immunity Guardian, Participates in Skin Urticarial Reactions and Contact Hypersensitivity

Naotomo Kambe[1], Yuumi Nakamura[1,2], Megumu Saito[3,4] and Ryuta Nishikomori[3]

ABSTRACT

Urticarial rash, one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS), is caused by a mutation in the gene encoding for *NLRP3* (nucleotide-binding oligomerization domain, leucine-rich repeats containing family, pyrin domain containing 3). This intracellular pattern recognition receptor and its adaptor protein, called apoptosis associated speck-like protein containing a caspase-recruitment and activating domain (ASC), participate in the formation of a multi-protein complex termed the inflammasome. The inflammasome is responsible for activating caspase-1 in response to microbial and endogenous stimuli. From the analysis of cellular mechanisms of urticarial rash in CAPS, we have traced caspase-1 activated IL-1β in CAPS to a surprising source: mast cells. Recently, two groups have generated gene-targeted mice that harbored *Nlrp3* mutations. These mice had very severe phenotypes, with delayed growth and the development of dermatitis, but not urticaria. The reason for the differences in the skin manifestations observed with CAPS and these knock-in mice relates to the findings that the inflammasome also plays a role in contact hypersensitivity, and that IL-18, another cytokine involved with inflammasome-activation of caspase-1, may be a major player in dermatitis development.

KEY WORDS

contact hypersensitivity, IL-1β, inflammasome, NLRP3, urticaria

INTRODUCTION

The skin is the primary interface between the interior of the body and the external environment. The skin functions to retain water, prevent the permeation or loss of other molecules and maintain body temperature. The skin also physically protects us from microbial invasion. Furthermore, it has become established that the skin itself plays a major role in the immune system.[1]

Recognition of invading microorganisms is essential for inducing an effective immune response. This process is mediated by germ line-encoded pattern recognition receptors, which can also be found in plants that do not have circulating white blood cells. To date, the most extensively studied pattern recognition receptors have been the Toll-like receptors (TLRs). Using their leucine-rich repeats (LRRs), these transmembrane proteins recognize conserved bacterial constituents, such as lipopolysaccharide (LPS). More recently, another class of pattern recognition receptors, called nucleotide-binding oligomerization domain (NOD)-LRRs containing family (NLRs), have been identified.[2] While TLRs detect bacterial products at the outer cell surface or in endosomes, intracellular NLRs mediate cytoplasmic recognition of bacterial products.[3]

Several NLR members participate in the formation of a multi-protein complex termed the inflammasome that is responsible for activating caspase-1 in re-

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Received 24 November 2009.

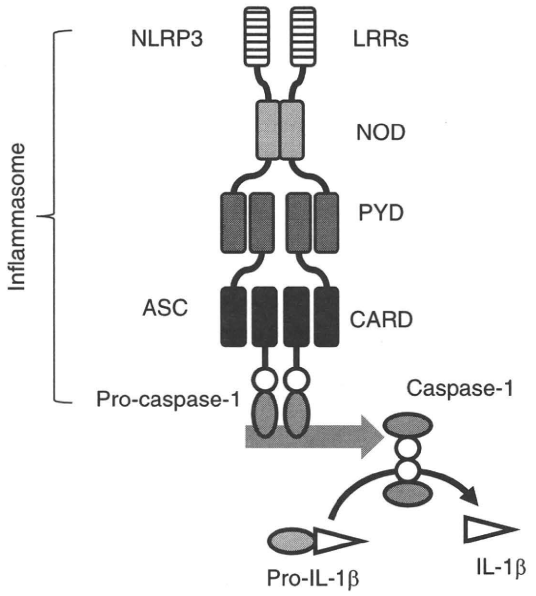

Fig. 1 The inflammasome is comprised of an NLR protein and an adaptor protein ASC, which can bridge the NLR to pro-caspase-1. Activation of the inflammasome results in self-cleavage and activation of pro-caspase-1 into the active protease. Then, activated caspase-1 cleaves its target molecules, including pro-IL-1β, into biologically active forms. LRRs, leucine-rich repeats; NOD, nucleotide-binding oligomerization domain; PYD, pyrin domain; CARD, caspase-recruitment and activating domain; NLRP3, NOD-LRRs containing PYD 3; ASC, apoptosis-associated speck-like protein containing a CARD.

sponse to microbial and endogenous stimuli.[4,5] The inflammasome is comprised of an NLR protein and an adaptor protein called apoptosis associated speck-like protein containing a caspase-recruitment and activating domain (ASC), which can bridge the NLR to pro-caspase-1 (Fig. 1). Activation of the inflammasome results in self-cleavage and activation of pro-caspase-1 into the active protease. Then, activated caspase-1 cleaves its target molecules, including pro-IL-1β, into biologically active forms.[6-9] IL-1β participates in systemic and local responses to infection, injury and immunological challenges by inducing fever, activating lymphocytes and by promoting leukocyte infiltrations at sites of injury or infection.[10,11]

In this review, we will discuss the functions of the inflammasome in the skin. In particular, we will focus on the inflammasome comprised of NLR family, pyrin domain containing 3 (NLRP3) in the regulation of IL-1β production and the implications for skin diseases.

URTICARIA

Urticaria, or hives, is a common disease that affects up to 20% of the general population at least once during their lifetimes.[12] This allergic disorder involving the skin is fleeting in nature, as it is characterized by the sudden appearance of wheals but the returning to its normal appearance without pigmentation, usually within 1-24 hours.[13] In its histological aspects, the wheal consistently exhibits localized edema of the dermis with dilatations of the post-capillary venules and lymphatic vessels. However, perivascular infiltrates show variable intensities comprised of neutrophils, and/or eosinophils, macrophages and T cells.[13] These inconsistent histological findings may underline the complex nature of the pathogenesis of urticaria, which has many sources including histamine release by activated mast cells.

Although patients with acute urticaria often complain of an upper airway infection, the eliciting cause is unclear in over 50% of patients. In particular, for chronic urticaria, defined as that persisting longer than 6 weeks, triggers remain unidentifiable in the majority of cases, despite extensive clinical and laboratory investigations.[12] In addition, urticaria is sometimes triggered by cold or heat contact, solar exposure, delayed pressure, mechanical stimuli and vibration. These subtypes of urticaria are classified as physical urticaria. Other types of urticaria are aquagenic, cholinergic, evoked by a contact irritant or exercise-induced.[13] Moreover, 2 or more different subtypes of urticaria can co-exist in any given patient.

Compared with the variety of proposed causes for urticaia, the strategy for treatment is relatively simple. Histamine H1-receptor antagonists are recommended as the first line of treatment because histamine release by cutaneous mast cells plays an important role in the development of urticaria. However, good responses using oral antihistamines have only been recorded for 40% of patients.[12] Antihistamines have been shown not to be effective for physical urticaria, suggesting that in a significant number of individuals, chronic urticaria is mediated via histamine-independent mechanisms.

CRYOPYRIN-ASSOCIATED PERIODIC SYNDROME

An urticarial rash (Fig. 2) that develops in the neonatal or early infant period is one of the clinical manifestations characteristic of cryopyrin-associated periodic syndrome (CAPS). CAPS is caused by a mutation in the gene encoding for *NLRP3*, previously known as *NALP3/CIAS1*/cryopyrin.[14,15] CAPS includes a spectrum of hereditary periodic fever disorders that comprise 3 phenotypically overlapping, but relatively distinct syndromes: familial cold antoinflammatory syndrome [FCAS, Mendelian inheritance in men number (MIM) #120100], Muckle-Wells syndrome (MWS, MIM #191900) and chronic infantile neurological cutaneous and articular (CINCA) syndrome (MIM #607115), also known as neonatal-onset multisystem inflammatory disease. FCAS and MWS are characterized by periodic attacks of urticarial rash, fever and arthralgia; whereas patients with CINCA syndrome,

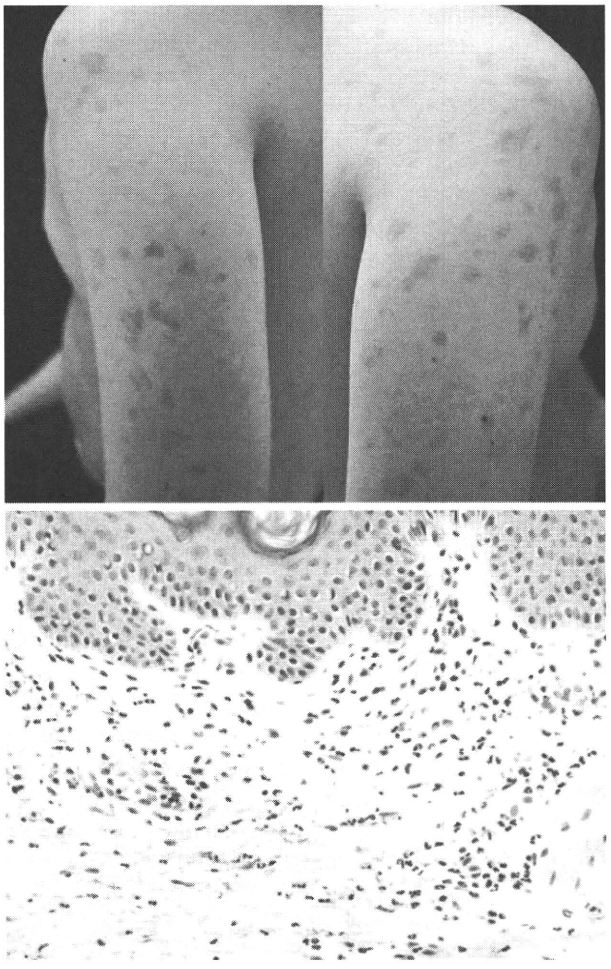

Fig. 2 Urticarial rash in a CAPS patient with an R260W mutation in *NLRP3*. The eruption is non-pruritic and returns to its normal appearance without pigmentation. Histopathological sample from a skin eruption shows edema in the dermis accompanied by the perivascular infiltration of inflammatory cells, predominantly neutrophils.

the most severe form of CAPS, exhibit chronic urticaria, as well as fever, arthropathy, chronic meningitis, papilledema, growth and mental retardation and hearing loss (Table 1).[16] The urticarial rash observed in CAPS is similar to that associated with common urticaria. However, unlike ordinary urticaria, the rash observed in most CAPS patients is not pruritic and responds to therapy with an IL-1 receptor antagonist rather than antihistamines.[17-19]

INFLAMMASOME ACTIVATION IN MAST CELLS

After development of an anti-IL-1β specific antibody (canakinumab), it was found that patients with CAPS have abnormally high levels of circulating IL-1β, approximately 5 times the normal amount.[20] In addition, treating patients with canakinumab relieved their

Table 1 Clinical manifestations of cryopyrin-associated periodic syndromes

	FCAS	MWS	CINCA
Skin rash	+	++	+++
Joint involvement	Arthritis?	Arthritis/ Arthralgia	Arthritis/ Arthralgia
Cartilage target	-	-	+++
Deafness	-	++	+++
Chronic meningitis	-	+?	+++
Eye involvement	-	+	+++
Amyloidosis	+	++	?

FCAS, familial cold auto-inflammatory syndrome; MWS, Muckle-Wells syndrome; CINCA, chronic infantile neurological, cutaneous and articular syndrome.

rashes within a day, suggesting that IL-1β was the sole cytokine responsible for the skin eruptions in CAPS. The disease-responsible gene, *NLRP3*, is predominantly expressed in monocytes, granulocytes and chondrocytes.[14] However, after performing immunohistochemical staining, we traced IL-1β in CAPS to a surprising source: mast cells.[21] Interestingly, mast cells in the skin samples from CAPS patients expressed active IL-1β without any treatment, whereas those from healthy donors only expressed active cytokine when appropriately stimulated.

Primary mast cells derived from mouse bone marrow and human cord blood expressed inflammasome components, including *NLRP3* and its adapter protein *ASC*. As was the case with macrophages, production of mature IL-1β via the NLRP3-inflammasome in mast cells required 2 signals. Microbial ligands, such as LPS, trigger the synthesis of the IL-1β precursor. A second ATP-triggered signal activates the inflammasome. Although a major function of LPS is to induce pro-IL-1β production, LPS also promotes the expression of *Nlrp3* in mast cells.

In macrophages, ATP-driven stimulation via the purinergic receptor P2X, ligand-gated ion channel 7 (P2RX7) is essential for caspase-1 proteolytic cleavage and IL-1β secretion by LPS-primed cells (Fig. 3).[22,23] P2RX7 forms a non-selective ion channel upon activation with ATP and, after stimulation, mediates K^+ efflux, which may be important for activating the inflammasome.[24] This ion channel mediated by P2RX7 rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore that allows passage of molecules as large as 900 Da.[22] It is possible, as has been proposed for macrophages,[25] that ATP promotes passage of microbial ligands, such as LPS, via pannexin-1 to trigger inflammasome activation in mast cells. Consistent with this conjecture, ATP alone did not induce IL-1β secretion by mast cells, even though ATP triggered large-pore formation. IL-1β secretion by mast cells was blocked for cells derived from *P2rx7*-deficient mice or by incubation in high K^+

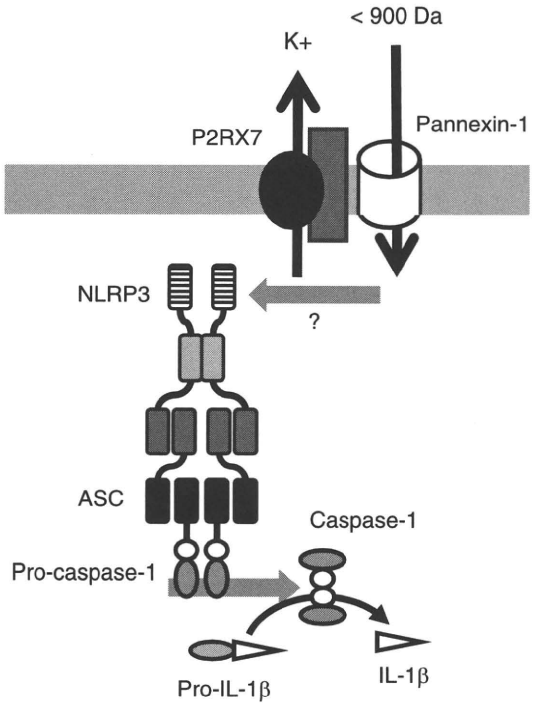

Fig. 3 P2RX7 forms a non-selective ion channel upon activation by ATP. Upon stimulation, P2R7X mediates K+ efflux and rapidly transforms to a pore-like structure by recruiting a pannexin-1 pore, which allows passage of molecules as large as 900 Da.

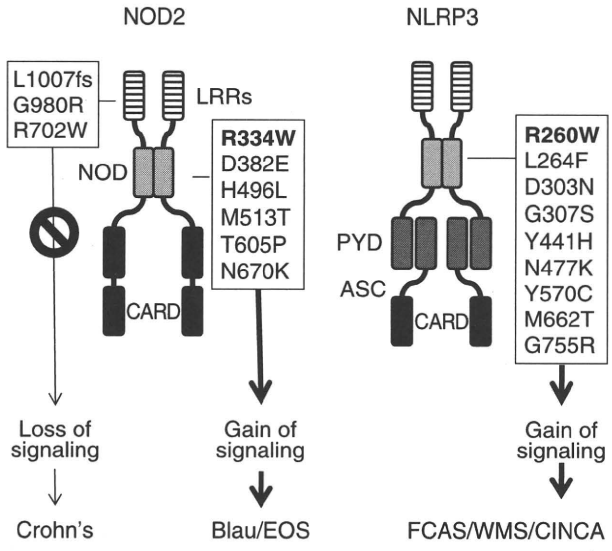

Fig. 4 Autoinflammatory disease-associated mutations in NOD2 and NLRP3. Crohn's disease-associated mutations found primarily in the LRRs of NOD2 result in a loss of activity, whereas mutations associated with Blau syndrome and early onset sarcoidosis (EOS) clustered in the NOD region of NOD2 cause constitutive activation of the molecule. Uncontrolled constitutive activation of NLRP3 in FCAS, MWS and CINCA are found in the NOD region of NLRP3. The mutations in NOD2 and NLRP3 associated with autoinflammatory diseases shown here are those found from our experience with cases and those mutations shown in bold are in analogous positions in the two proteins of NOD2 and NLRP3.

extracellular medium. Thus, ATP-driven P2RX7 and K^+ efflux are also required for effective IL-1β secretion by mast cells.[21]

CAPS-ASSOCIATED NLRP3 INDUCES URTICARIA

Disease-associated mutations associated with CAPS are localized to the centrally located NOD region in *NLRP3*. Of note, similar missense mutations in *NOD2* have been identified in patients with Blau syndrome, another autosomal-dominant autoinflammatory syndrome, and early-onset sarcoidosis, a set of sporadic granulomatous disorders that phenotypically resemble Blau syndrome.[26-28] Interestingly, the amino acids affected by an R260W mutation in *NLRP3* and an R334W mutation in *NOD2* are at analogous sequence positions, suggesting a common molecular mechanism for their roles in the development of autoinflammatory disease (Fig. 4). Via molecular interactions of their LRRs with their own NOD regions, NLRP3 and NOD2 are maintained in inactive conformations. This is relieved by ligand recognition via the LRRs.[29,30] Disease-associated mutations are thought to mimic active conformational changes that are induced by microbial ligands, and *in vitro* studies suggest that these mutations exert gain-of-function effects.[5,29] In the case of NLRP3, in addition of NF-κB activation, the mutation found in CAPS constitutively activates caspase-1 to produce active IL-1β.

Mouse *Nlrp3* mutants, corresponding to those observed in human CAPS, induced constitutive *Asc*-dependent NF-κB activation and IL-1β secretion.[21] Transfer of mast cells expressing an R258W mutant, corresponding to R260W of human CAPS-associated NLRP3, induced perivascular neutrophil-rich inflammation in mouse skin, a histological hallmark of the urticaria observed in CAPS patients. These findings are consistent with a previous report that showed enhanced production of IL-1β in the skin of CAPS patients.[18]

However, it remains unclear why mast cells with a constitutively activated NLRP3-inflammasome produce mature IL-1β, even in the absence of LPS. One possibility is that CAPS-associated NLRP3 mutants induce pro-IL-1β via constitutive activation of NF-κB induction.[31,32] Another possibility is that production of pro-IL-1β is induced by endogenous or environmental cues that operate in the skin independently of NLRP3. Consistent with this latter model, the characteristic skin rash observed in CAPS often develops within the first few weeks of life when the skin is first

exposed to environmental factors. This may include exposure to small amounts of LPS and/or other microbial stimuli. The observation that skin abnormalities in incontinentia pigmenti (MIM #308300) commence at birth[33] is also consistent with this possibility. This disorder is an X-linked dominant inherited disorder caused by a mutation of NEMO, a gene that encodes the regulatory component of the I-κB kinase complex responsible for activating the NF-κB signaling pathway.

Collectively, inflammasome activation in mast cells contributes to the pathogenesis of IL-1β-mediated diseases of the skin. Mast cells reside in numerous tissues and also participate in experimental models of arthritis[34] and encephalomyelitis.[35,36] Thus, it is possible that these cells play a role in disease pathogenesis not only in the skin, but also in the joints and central nervous system, which are also major diseased sites in CAPS patients.[16] Additional studies are needed in order to better understand the contributions of mast cells to IL-1β-mediated diseases associated with NLRP3.

Furthermore, urticaria that is associated with CAPS is usually non-pruritic and unresponsive to antihistamines. This clinical observation is in line with experimental results that NLRP3-inflammasome activation induces IL-1β secretion, but not degranulation, in mast cells. Nonetheless, mast cells that expressed a CAPS-associated NLRP3 mutant promoted vascular permeability, a cellular response critical for wheal formation *in vivo*. Because many cases of non-CAPS urticaria are unresponsive to histamine H1-receptor antagonists, it is possible that skin rash associated with histamine resistance is mediated via inflammasome activation in mast cells. Thus, understanding the pathophysiology of CAPS may provide critical insights into more common diseases, such as antihistamine-refractory urticaria.

CAPS AND KNOCK-IN MICE

Recently, two groups[37,38] have generated gene-targeted mice harboring *Nlrp3* mutations that mimic the amino acid substitutions in NLRP3 that were found to cause disease susceptibility in CAPS. The mice generated in both studies had very severe phenotypes. Newborn mice with an R258W mutation in *Nlrp3* exhibited delayed growth, decreased weight gain and increased mortality. Adult animals were infertile and developed dermatitis, but not urticaria, associated with increased sizes of lymphoid organs.[38] The mice with A350V in *Nlrp3* died between days 2 and 14 and exhibited profound growth delays, skin abscesses, and hair growth and pigmentation defects.[37]

At the cellular level, despite the observation that T cells in mutant mice displayed altered polarization profiles favorable to Th17, both groups found that this defect was due to the expression of the mutated *Nlrp3* in antigen-presenting cells (APCs), but not in T cells. They isolated bone marrow-derived macrophages or dendritic cells (DCs) and explored their responses to TLR ligands in the presence or absence of ATP. In contrast to a study of CD14-positive peripheral mononuclear cells from a human CAPS patient that secreted IL-1β without any treatment,[30] myeloid cells from mutant mice in both studies did not spontaneously secrete mature IL-1β. Rather, they displayed a considerably higher sensitivity to TLR ligands than cells from wild type (WT) mice.[37,38] Importantly, for optimal activation, addition of exogenous ATP was not required in order to release IL-1β, supporting the notion that the mutated Nlrp3 was constitutively active and, therefore, did not require additional stimuli for inflammasome activation.

An important outcome from these knock-in mice was that a common feature evoked by CAPS-associated *Nlrp3* was the development of severe skin lesions. These included erythema, abscesses, scaling and thickening of both the epidermis and dermis. However, these observations only recapitulated some, but not all, of the urticaria-like skin lesions reported in CAPS patients. Moreover, both studies still failed to address the critical question of whether or not TLR-dependent accumulation of pro-IL-1β was necessary for the burst of IL-1β secretion in CAPS.

CONTACT HYPERSENSITIVITY

The reasons for the discrepancies between the skin manifestations of CAPS and gene-targeted mice harboring the *Nlrp3* mutations could be directed to findings that the inflammasome also plays a role in contact hypersensitivity. Contact hypersensitivity involves the priming of naïve T cells after sensitizing chemicals penetrate the skin surface (sensitization) and primed T cells are activated upon re-exposure to the antigen (elicitation). Elicitation can be further subdivided into early and late phases.[39] The early phase, characterized by increased vessel permeability and local edema, peaks 8 hours after antigen re-exposure and is believed to be mediated by local release of mediators, including IL-1β and histamine.[40] The late phase develops 12-36 hours after antigen re-exposure and is due to cellular infiltration. During these processes, DC migration, antigen presentation, expansion of specific T cells and recruitment of T cells to the skin depend on the coordinated interactions of inflammatory cytokines, namely IL-1β and IL-18,[41] which are important cytokines for initiating specific T-cell-mediated immune responses.

A role for the inflammasome in contact hypersensitivity was analyzed by Watanabe *et al.*[42] who found that key components of the inflammasome were present in keratinocytes. Some contact sensitizers, such as trinitrochlorobenzene (TNCB), can induce caspase-1-mediated cleavage and activation of IL-1β and IL-18 in an ASC-dependent manner. Interestingly,

chemical irritants like SDS and physical agents like ultraviolet B could also trigger inflammasome activation in keratinocytes.[43,44] Subsequently, it was found that mice lacking *Nlrp3* and adaptor protein *Asc*-deficient mice had impaired early phase reactions during the challenges. Thus, the inflammasome can modulate the early effector phase of T cell-mediated immune responses.

These findings, however, are in contrast with a report by Sutterwala *et al.*[45] who found that impaired responses in *Nlrp3*- and *Asc*-deficient mice were seen in late-phase. Interestingly, in this report, *Nlrp3*-deficient mice that received cells from sensitized WT animals showed almost normal ear swelling, whereas WT mice that received cells from sensitized donors lacking *Nlrp3* failed to develop ear swelling. This suggests that Nlrp3 is necessary for inducing antigen-specific T-cell responses. Indeed, Langerhans cell migration and an optimal contact hypersensitivity response require functional caspase-1,[46] even though recent data suggest that important cross-presenting APCs in the skin are not Langerhans cells, but are langerin$^+$/CD103$^+$ DCs, most likely of dermal origin.[47,48]

NECROSIS AND NLRP3

How can we explain the discrepancies in these two reports on contact hypersensitivity in mice? One possibility is that TNCB is very potent when causing direct tissue damage, and that the signaling involved might be strong enough to overcome the requirement of inflammasome activation for T-cell priming.[49]

Of potential interest is a recent report showing that necrosis directly activates the NLRP3 inflammasome.[50,51] By treating human monocytic THP-1 cells with indirubin oxime derivative 7-bromoindirubin-3'-oxime, Li *et al.*[50] showed that the NLRP3 inflammasome was activated in cells undergoing necrosis, resulting in the production of mature IL-1β and IL-18. It is remarkable that inflammasome activation and the release of these caspase-1 targeting cytokines did not require LPS priming or other pro-inflammatory stimuli.

Along these lines, the activation of NLRP3 itself mediates a form of necrosis termed pyronecrosis.[52,53] We previously showed that the expression of a disease-associated mutation of *NLRP3* resulted in a caspase-1 independent, but cathepsin B-dependent form of cell death.[32] This characteristic cell death can also be observed in monocytes derived from CAPS patients. NLRP3-mutant monocytes rapidly and selectively underwent necrosis-like programmed cell death after treatment with LPS accompanied by the induction of *NLRP3* expression. This unique NLRP3 phenotype enabled us to differentiate NLRP3-mutated cells from WT cells in CAPS patients who had disease-associated mutant *NLRP3* as a latent mosaicism.[54]

Pyronecrosis is caspase-independent; neither the activating cleavage of effector caspase-3 nor its substrate poly-(adenosine diphosphate ribose) polymerase (PARP) occurs during cell death. Pyronecrosis proceeds in the presence of caspase-1-specific inhibitors, and even pan-caspase inhibitors. However, cell death is abrogated in the presence of CA074-Me, an inhibitor of the lysosomal protease cathepsin B, implicating the contribution of lysosome activity in the pathway. Pyronecrotic cells do not demonstrate DNA fragmentation or a loss of mitochondrial membrane potential. By electron microscopy, the morphological changes characteristic of pyronecrosis are consistent with necrosis and include membrane degradation and uncondensed chromatin. Similar to classic necrosis, pyronecrosis is accompanied by the release of the immune modulator high-mobility group box 1 (HMGB1). Pyronecrosis induced by NLRP3 activation suggests an exciting connection between cell death and inflammation in response to cellular insult to injury.

However, we should note that necrosis does not always induce inflammasome activation. Treatment with hydrogen peroxide or paclitaxel, which induce caspase-independent necrosis, fails to induce inflammasome activation. Similarly, induction of necroptosis through TNF-α in the presence of caspase-3 and caspase-9 inhibitors[55] did not result in inflammasome activation. If necrosis was induced too rapidly by repeated cycles of freeze-thaw or excessive osmotic shock, inflammasome activation was greatly reduced. Based on these findings, during activation of the NLRP3 inflammasome, the dissolution of the cellular architecture that occurs during some specific forms of necrosis may be required for lysosome destabilization.[56]

Another impressive point to remember from the study by Li *et al.*[50] is that it remains unclear how pro-IL-1β is induced in a sterile environment without bacterial components like LPS. Several endogenous danger signals released by necrotic cells have been shown to stimulate TLRs and other pattern recognition receptors and, therefore, have the potential to induce pro-IL-1β, although this has not been definitively demonstrated. Of note, endogenous molecules can serve a priming role for NLRP3-inflammasome activation.[51] Biglycan and hyaluronic acid, components of the extracellular matrix, were capable of priming macrophages for Nlrp3-inflammasome activation in response to pressure-disrupted necrotic cells. Hence, extracellular matrix components that accumulate in non-physiological sites or amounts can function as signal for the induction of pro-IL-1β accumulation, suggesting that inflammasome activation can occur *in vivo* in sterile settings without microbes.

Another possibility is that inflammasome activation during necrosis can lead to the release of mature IL-18, even in the absence of microbial stimuli, as IL-18

is constitutively expressed by several cell types. IL-18, in turn, via activation of the MyD88-dependent pathway, leads to the transcription of pro-inflammatory cytokine genes, including IL-1β,[57] and amplifies inflammation. Thus, IL-18 may be one of the earliest mediators of the sterile inflammatory response that is triggered by necrosis or tissue damage.

IL-18 AND DERMATITIS

In a report by Watanabe *et al.*,[42] IL-1 receptor deficiency resulted in a significant decrease in the intensity of ear swelling. However, it did not totally abrogate ear swelling after elicitation with TNCB, suggesting that other cytokines/signals are likely to modulate the early phases of contact hypersensitivity. This may be due, at least in part, to the presence of IL-18, which is also activated by caspase-1.[58,59]

Keratinocytes constitutively produce both pro-IL-1β[60] and pro-IL-18,[61] but lack endogenous caspase-1 activity under normal conditions.[60] Using established keratinocyte-specific caspase1-transgenic mice with a human keratin 14 promoter that specifically expressed the targeted gene at the basal layer of keratinocytes, Yamanaka *et al.*[62] showed that the mice spontaneously suffered from chronic dermatitis under specific pathogen-free (SPF) conditions, which was accompanied by abnormally elevated serum levels of IL-18 and IL-1β. Another transgenic mouse model in which epidermal cells over secreted IL-18 also spontaneously developed atopic dermatitis-like skin eruption under SPF conditions, and a deletion of *Il18* protected against the development of skin eruptions.[63] This finding suggests that excessive cutaneous IL-18 release is a causative factor for the development of dermatitis. Of potential interest is that the phenotypes in the skin of these caspase-1 and IL-18 transgenic mice closely resembled that in disease-associated *Nlrp3* knock-in mice.

Furthermore, Terada *et al.*[64] developed an intrinsic atopic dermatitis mouse model with daily applications of protein A, a surface molecule and virulence factor of *Staphylococcus aureus*, which resulted in destruction of the skin barrier with a subclinical dose of SDS. In this model, neutralizing anti-IL-18 antibodies and *Il18*-deficient mice could completely protect against SDS plus *S. aureus*-derived protein A-induced dermatitis, suggesting the importance of IL-18 for atopic dermatitis-like skin eruptions. This is also interesting when we consider the recent findings that hemolysins and bacterial lipoproteins in *S. aureus* can induce activation of the NLRP3 inflammasome.[65,66] Thus, another target inflammasome-activating cytokine, IL-18, may be a major player for dermatitis development.

LESSONS FROM CAPS

During the past decade, our understanding of the cellular and molecular mechanisms by which innate immune system molecules sense specific molecular patterns of components of invading organisms has increased tremendously. The NLRs, together with the TLRs, are now appreciated as parts of this important sensing system that allows the host to generate effective immune responses. NLRP3 also detects various endogenous, sterile danger signals in the absence of microbial infection. Progress in understanding the roles of NLRs will improve our knowledge to answer questions, such as how to transfer insights from mouse model systems to translational research focusing on human pathology, especially from the rare genetic disorder CAPS, associated with an *NLRP3* mutation, to more common diseases, such as ordinary urticaria and dermatitis. Interestingly, two NLRP3 single nucleotide polymorphisms (SNP, rs4612666 and rs10754558) were recently reported to be significantly associated with susceptibility to food-induced anaphylaxis as well as aspirin-induced asthma.[67] Functional analysis of the rs4612666 SNP located in intron 7 of *NLRP3* showed 1.2-fold higher transcriptional enhancer activity than the other constructs containing the T allele, whereas rs10754558 in the 3'untranslated region affected the stability of the *NLRP3* mRNA. Thus, NLRP3 polymorphisms may increase the risk of the hypersensitive phenotype of allergy.

However, we still cannot answer all of the questions for the roles of the inflammasome in the skin. IL-18, as well as IL-33, are target molecules for inflammasome-activated caspase-1. Nevertheless, why does the urticarial rash in CAPS depend solely on IL-1β? Why can't we induce hives in mice, even if activated *Nlrp3* is induced? We should still look carefully at the clinical manifestations of CAPS in order to determine what happens in humans when the inflammasome is activated.

ACKNOWLEDGEMENTS

We thank Akihiro Fujisawa, Hideaki Tanizaki, Yoshiki Miyachi, and Tatsutoshi Nakahata (Kyoto University), Makoto Murakami (The Tokyo Metropolitan Institute of Medical Science), Yun-Gi Kim and Gabriel Núñez (The University of Michigan), Shinji Shimada (Yamanashi University) and Hiroyuki Matsue (Chiba University) for their encouragement and support of the work on NLRs. We also wish to express our sincere thanks to the CAPS patients, their parents and physicians for their participation in our studies.

This work was supported, in part, by Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (N.K., M.S. and R.N.), The Cell Science Research Foundation (Y.N.) and KANAE Foundation for the Promotion of Medical Science (Y.N.).

REFERENCES

1. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009;**9**:679-91.
2. Ting JP, Lovering RC, Alnemri ES *et al.* The NLR gene family: a standard nomenclature. *Immunity* 2008;**28**:285-7.
3. Ye Z, Ting JP. NLR, the nucleotide-binding domain leucine-rich repeat containing gene family. *Curr Opin Immunol* 2008;**20**:3-9.
4. Mariathasan S, Newton K, Monack DM *et al.* Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 2004;**430**:213-8.
5. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002;**10**:417-26.
6. Church LD, Cook GP, McDermott MF. Primer: inflammasomes and interleukin 1beta in inflammatory disorders. *Nat Clin Pract Rheumatol* 2008;**4**:34-42.
7. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol* 2009;**27**:229-65.
8. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 2007;**14**:10-22.
9. Petrilli V, Dostert C, Muruve DA, Tschopp J. The inflammasome: a danger sensing complex triggering innate immunity. *Curr Opin Immunol* 2007;**19**:615-22.
10. Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996;**87**:2095-147.
11. Lamkanfi M, Dixit VM. Inflammasomes: guardians of cytosolic sanctity. *Immunol Rev* 2009;**227**:95-105.
12. Nettis E, Pannofino A, D'Aprile C, Ferrannini A, Tursi A. Clinical and aetiological aspects in urticaria and angioedema. *Br J Dermatol* 2003;**148**:501-6.
13. Zuberbier T, Asero R, Bindslev-Jensen C *et al*, and Dermatology Section of the European Academy of Allergology and Clinical Immunology, Global Allergy and Asthma European Network, European Dermatology Forum, World Allergy Organization. EAACI/GA (2) LEN/EDF/WAO guideline: definition, classification and diagnosis of urticaria. *Allergy* 2009;**64**:1417-26.
14. Feldmann J, Prieur AM, Quartier P *et al.* Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am J Hum Genet* 2002;**71**:198-203.
15. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* 2001;**29**:301-5.
16. Neven B, Prieur AM, Quartier dit Maire P, and Medscape. Cryopyrinopathies: update on pathogenesis and treatment. *Nat Clin Pract Rheumatol* 2008;**4**:481-9.
17. Hawkins PN, Lachmann HJ, McDermott MF. Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N Engl J Med* 2003;**348**:2583-4.
18. Hoffman HM, Rosengren S, Boyle DL *et al.* Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 2004;**364**:1779-85.
19. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB *et al.* Use of canakinumab in the cryopyrin-associated periodic syndrome. *N Engl J Med* 2009;**360**:2416-25.
20. Lachmann HJ, Lowe P, Felix SD *et al.* In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med* 2009;**206**:1029-36.
21. Nakamura Y, Kambe N, Saito M *et al.* Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. *J Exp Med* 2009;**206**:1037-46.
22. Ferrari D, Pizzirani C, Adinolfi E *et al.* The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* 2006;**176**:3877-83.
23. Mariathasan S, Weiss DS, Newton K *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 2006;**440**:228-32.
24. Pelegrin P, Surprenant A. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* 2006;**25**:5071-82.
25. Kanneganti TD, Lamkanfi M, Kim YG *et al.* Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* 2007;**26**:433-43.
26. Kanazawa N, Okafuji I, Kambe N *et al.* Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome. *Blood* 2005;**105**:1195-7.
27. Miceli-Richard C, Lesage S, Rybojad M *et al.* CARD15 mutations in Blau syndrome. *Nat Genet* 2001;**29**:19-20.
28. Okafuji I, Nishikomori R, Kanazawa N *et al.* Role of the NOD2 genotype in the clinical phenotype of Blau syndrome and early-onset sarcoidosis. *Arthritis Rheum* 2009;**60**:242-50.
29. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 2004;**20**:319-25.
30. Saito M, Fujisawa A, Nishikomori R *et al.* Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome. *Arthritis Rheum* 2005;**52**:3579-85.
31. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G. Cryopyrin-induced interleukin 1beta secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. *J Biol Chem* 2004;**279**:21924-8.
32. Fujisawa A, Kambe N, Saito M *et al.* Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood* 2007;**109**:2903-11.
33. Berlin AL, Paller AS, Chan LS. Incontinentia pigmenti: a review and update on the molecular basis of pathophysiology. *J Am Acad Dermatol* 2002;**47**:169-87; quiz 188-90.
34. Nigrovic PA, Binstadt BA, Monach PA *et al.* Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1. *Proc Natl Acad Sci U S A* 2007;**104**:2325-30.
35. Dietsch GN, Hinrichs DJ. The role of mast cells in the elicitation of experimental allergic encephalomyelitis. *J Immunol* 1989;**142**:1476-81.
36. Robbie-Ryan M, Tanzola MB, Secor VH, Brown MA. Cutting edge: both activating and inhibitory Fc receptors expressed on mast cells regulate experimental allergic encephalomyelitis disease severity. *J Immunol* 2003;**170**:1630-4.
37. Brydges SD, Mueller JL, McGeough MD *et al.* Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity* 2009;**30**:875-87.

38. Meng G, Zhang F, Fuss I, Kitani A, Strober W. A mutation in the Nlrp3 gene causing inflammasome hyperactivation potentiates Th17 cell-dominant immune responses. *Immunity* 2009;**30**:860-74.
39. Grabbe S, Schwarz T. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 1998;**19**:37-44.
40. Kitagaki H, Ono N, Hayakawa K, Kitazawa T, Watanabe K, Shiohara T. Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. *J Immunol* 1997;**159**:2484-91.
41. Shornick LP, De Togni P, Mariathasan S *et al.* Mice deficient in IL-1beta manifest impaired contact hypersensitivity to trinitrochlorobenzone. *J Exp Med* 1996;**183**:1427-36.
42. Watanabe H, Gaide O, Petrilli V *et al.* Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 2007;**127**:1956-63.
43. Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD. The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr Biol* 2007;**17**:1140-5.
44. Keller M, Ruegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 2008;**132**:818-31.
45. Sutterwala FS, Ogura Y, Szczepanik M *et al.* Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* 2006;**24**:317-27.
46. Antonopoulos C, Cumberbatch M, Dearman RJ, Daniel RJ, Kimber I, Groves RW. Functional caspase-1 is required for Langerhans cell migration and optimal contact sensitization in mice. *J Immunol* 2001;**166**:3672-7.
47. Bedoui S, Whitney PG, Waithman J *et al.* Cross-presentation of viral and self antigens by skin-derived CD 103+ dendritic cells. *Nat Immunol* 2009;**10**:488-95.
48. Waithman J, Allan RS, Kosaka H *et al.* Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. *J Immunol* 2007;**179**:4535-41.
49. Yazdi AS, Ghoreschi K, Rocken M. Inflammasome activation in delayed-type hypersensitivity reactions. *J Invest Dermatol* 2007;**127**:1853-5.
50. Li H, Ambade A, Re F. Cutting edge: Necrosis activates the NLRP3 inflammasome. *J Immunol* 2009;**183**:1528-32.
51. Iyer SS, Pulskens WP, Sadler JJ *et al.* Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 2009;**106**:20388-93.
52. Ting JP, Willingham SB, Bergstralh DT. NLRs at the intersection of cell death and immunity. *Nat Rev Immunol* 2008;**8**:372-9.
53. Willingham SB, Bergstralh DT, O'Connor W *et al.* Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe* 2007;**2**:147-59.
54. Saito M, Nishikomori R, Kambe N *et al.* Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. *Blood* 2008;**111**:2132-41.
55. Hitomi J, Christofferson DE, Ng A *et al.* Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 2008;**135**:1311-23.
56. Hornung V, Bauernfeind F, Halle A *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 2008;**9**:847-56.
57. Puren AJ, Fantuzzi G, Gu Y, Su MS, Dinarello CA. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J Clin Invest* 1998;**101**:711-21.
58. Kawase Y, Hoshino T, Yokota K *et al.* Exacerbated and prolonged allergic and non-allergic inflammatory cutaneous reaction in mice with targeted interleukin-18 expression in the skin. *J Invest Dermatol* 2003;**121**:502-9.
59. Wang B, Feliciani C, Howell BG *et al.* Contribution of Langerhans cell-derived IL-18 to contact hypersensitivity. *J Immunol* 2002;**168**:3303-8.
60. Mizutani H, Black R, Kupper TS. Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. *J Clin Invest* 1991;**87**:1066-71.
61. Stoll S, Muller G, Kurimoto M *et al.* Production of IL-18 (IFN-gamma-inducing factor) messenger RNA and functional protein by murine keratinocytes. *J Immunol* 1997;**159**:298-302.
62. Yamanaka K, Tanaka M, Tsutsui H *et al.* Skin-specific caspase-1-transgenic mice show cutaneous apoptosis and pre-endotoxin shock condition with a high serum level of IL-18. *J Immunol* 2000;**165**:997-1003.
63. Konishi H, Tsutsui H, Murakami T *et al.* IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci U S A* 2002;**99**:11340-5.
64. Terada M, Tsutsui H, Imai Y *et al.* Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by Staphylococcus aureus product in mice. *Proc Natl Acad Sci U S A* 2006;**103**:8816-21.
65. Craven RR, Gao X, Allen IC *et al.* Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 2009;**4**:e7446.
66. Munoz-Planillo R, Franchi L, Miller LS, Nunez G. A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the Nlrp3 inflammasome. *J Immunol* 2009;**183**:3942-8.
67. Hitomi Y, Ebisawa M, Tomikawa M *et al.* Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. *J Allergy Clin Immunol* 2009;**124**:779-85. e6.

(1–3). In the first report by Barton et al (1), the IVIG infusion was followed by a decrease in IgG and IgM serum levels within 72 hours, and a typical biopsy-proved cryoglobulinemic membranoproliferative glomerulonephritis. In a case described by Odum et al (2), IVIG infusion was followed within 48 hours by diffuse purpura, a rise in plasma creatinine levels, a microscopic hematuria, and high-level proteinuria strongly suggestive of glomerulonephritis. Yebra et al reported a flare of hepatitis C virus–related cryoglobulinemic vasculitis 4 hours after the first IVIG infusion, an increase of cryoglobulin precipitation, and depletion of the monoclonal IgMκ after in vitro addition of IVIG, and suggested that this simple method could help to predict the risk of cryoglobulin–IVIG immune complex formation and should be performed before starting IVIG in patients with mixed cryoglobulinemia (3). As with infliximab and rituximab, we have reported in our article that polyvalent exogenous human immunoglobulins were also recognized in vitro by RF-positive IgMκ.

Taken together, these results strongly suggest that the recognition of monoclonal or polyclonal immunoglobulin by RF-positive IgMκ is not specific and that treating RF-positive IgMκ cryoglobulinemic vasculitis with either monoclonal immunoglobulins (e.g., rituximab or infliximab) or polyvalent immunoglobulins is associated with a risk of increased cryoprecipitation and vasculitis flare shortly after treatment initiation.

We believe that, in the presence of RF-positive IgMκ type II cryoglobulinemic vasculitis, any treatment with IVIG should be used with caution. IVIG does not have the clear benefit of rituximab in cryoglobulinemic vasculitis, and there is not a rationale for the use of monoclonal anti–tumor necrosis factor α antibodies. The use of rituximab, should, as well, be proposed cautiously in patients with a RF-positive IgMκ type II cryoglobulinemic vasculitis. We recommend that plasma exchanges should be performed to reduce high serum cryoglobulin levels, and that rituximab should be given in low doses. This precaution should also be recommended for other treatments that are based on B cell–depleting monoclonal antibodies, which have not yet been used in cryoglobulinemic vasculitis, such as veltuzumab (anti-CD20), inotuzumab ozogamicin, and epratuzumab (anti-CD22).

Dr. Cacoub has received consulting fees and honoraria from Bristol-Myers Squibb, Sanofi-Aventis, Gilead, Schering-Plough (less than $10,000 each), Roche, and Servier (more than $10,000 each).

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1. Barton JC, Herrera GA, Galla JH, Bertoli LF, Work J, Koopman WJ. Acute cryoglobulinemic renal failure after intravenous infusion of gamma globulin. Am J Med 1987;82:624–9.
2. Odum J, D'Costa D, Freeth M, Taylor D, Smith N, MacWhannell A. Cryoglobulinaemic vasculitis caused by intravenous immunoglobulin treatment. Nephrol Dial Transplant 2001;16:403–6.
3. Yebra M, Barrios Y, Rincon J, Sanjuan I, Diaz-Espada F. Severe cutaneous vasculitis following intravenous infusion of gammaglobulin in a patient with type II mixed cryoglobulinemia. Clin Exp Rheumatol 2002;20:225–7.

DOI 10.1002/art.27619

Enhanced NF-κB activation with an inflammasome activator correlates with activity of autoinflammatory disease associated with *NLRP3* mutations outside of exon 3: comment on the article by Jéru et al

To the Editor:

We wish to comment on a recent report regarding functional consequences of *NLRP3* mutations (1). Based on the genetic analysis of a family with atypical autoinflammatory symptoms, Jéru et al identified a mutation in the leucine-rich repeat (LRR) domain of *NLRP3*. *NLRP3* is a 9-exon gene comprising 3 major domains: an amino-terminal pyrin domain, a nucleotide-binding oligomerization domain (NOD), and carboxyl-terminal LRR. More than 50 disease-associated mutations have been described for *NLRP3*; most were found within the centrally located NOD encoded by exon 3.

Jéru et al reported that NLRP3 was an inhibitory molecule, although the function of NLRP3 in NF-κB signaling remains controversial. We previously reported that *NLRP3* mutations showed spontaneous ASC-dependent NF-κB activation, and this was clearly associated with disease severity in patients with cryopyrin-associated periodic syndrome (CAPS) (2). However, among 11 mutations identified from our recruited patients (3), G755R located in exon 4 did not show spontaneous NF-κB activation (Figure 1A), even though a patient carrying G755R had severe disease manifestations. To date, 2 other *NLRP3* mutations located outside of exon 3 have been reported: G755A (4) and Y859C (5). G755A was identified in a typical CAPS patient (4). In contrast, a patient with Y859C had only 1 episode of a transient rash and, of note, absence of fever (5). A family member with Y859C (1) also did not manifest skin eruptions and showed a relatively mild phenotype. As seen in Figure 1A, neither G755A in exon 4 nor Y859C in exon 6 exhibited NF-κB activation.

For *NOD2*, gain-of-function mutations associated with granulomatous disorders are recognized in the centrally located *NOD* and exhibit similar spontaneous activation of NF-κB without the *NOD2* ligand (6). Interestingly, the amino acids affected by an R260W mutation in *NLRP3* and an R334W mutation in *NOD2* are at analogous positions, suggesting a common molecular mechanism for development of autoinflammatory disease. In contrast, *NOD2* mutations at LRRs, related to Crohn's disease, show defective responses to the *NOD2* ligand. In the presence of R837, an NLRP3-inflammasome activator, a G755R mutation located outside exon 3 of *NLRP3* showed remarkably enhanced NF-κB activation with an activity level that was higher than that observed with the R260W mutation (Figure 1B). With regard to 2 other mutations located outside of exon 3, G755A showed slightly increased NF-κB activation with R837, whereasY859C, which was identified in the atypical CAPS family whose members had mild phenotypes, did not. Thus, the enhanced NF-κB activation after stimulation with R837 correlates with disease activity, including mutations outside of exon 3. However, we still do not know how R837 activates the NLRP3 inflammasome.

We agree that *NLRP3* mutations, especially those identified from de novo cases, should be carefully evaluated by functional analyses. We believe that, in addition to excess production of interleukin-1β (IL-1β), enhanced NF-κB activa-

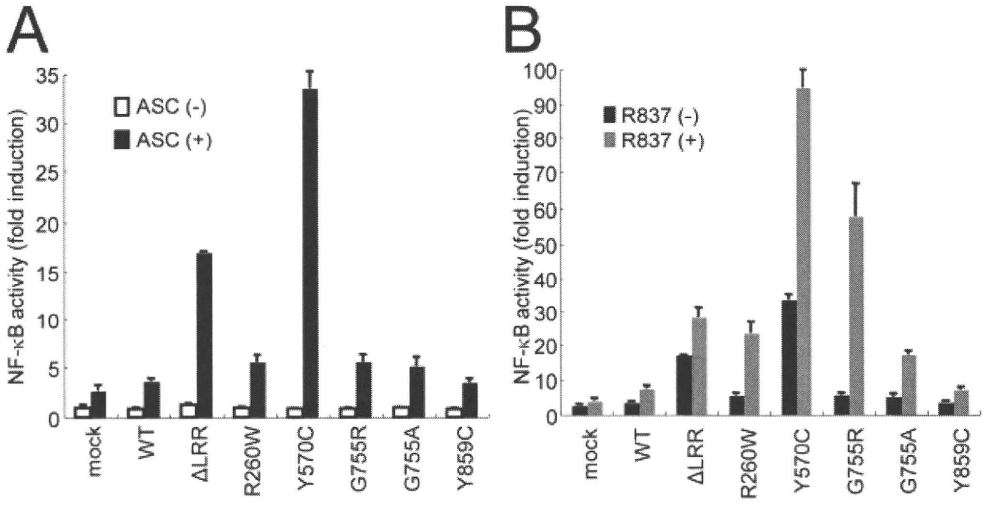

Figure 1. NF-κB activity in disease-associated mutations of *NLRP3*. **A,** Spontaneous NF-κB activation occurs in disease-associated mutations of *NLRP3*, but not in mutations located outside of exon 3. Expression plasmids for NLRP3, its deletion mutation lacking leucine-rich repeats (ΔLRRs), and ASC in the pEF-BOS vector background have been previously described (2). Mock is an empty vector. WT is wild-type NLRP3. R260W and Y570C are disease-associated mutations located within exon 3. G755R, G755A, and Y859C are the mutations outside of exon 3. **B,** Addition of R837 induces appreciable NF-κB activation in G755R mutations compared with WT *NLRP3*. ASC-dependent activation of NF-κB in the presence and absence of 10 μg/ml R837 was assessed. Values are the mean and SD of normalized data (in relation to mock with ASC [**A**] or R837 [**B**], set at 1), from triplicate cultures. Representative data from 3 independent analyses with similar results are shown.

tion may be associated with the accumulation of the IL-1β proform (7), which may also contribute to disease onset and the clinical manifestations of CAPS. In addition, careful observations of patients who bear *NLRP3* mutations outside of exon 3, who sometimes present with atypical symptoms of CAPS as in the cases described by Jéru et al, may provide an opportunity to better understand the physiologic and pathologic functions of NLRP3.

Supported in part by the Ministry of Education, Science, Sports, and Culture, and the Ministry of Health, Labor, and Welfare, Japan.

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1. Jeru I, Marlin S, Le Borgne G, Cochet E, Normand S, Duquesnoy P, et al. Functional consequences of a germline mutation in the leucin-rich repeat domain of NLRP3 identified in an atypical autoinflammatory disorder. Arthritis Rheum 2010;62:1176–85.
2. Fujisawa A, Kambe N, Saito M, Nishikomori R, Tanizaki H, Kanazawa N, et al. Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. Blood 2007;109:2903–11.
3. Saito M, Nishikomori R, Kambe N, Fujisawa A, Tanizaki H, Takeichi K, et al. Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. Blood 2008;111:2132–41.
4. Aksentijevich I, Remmers EF, Goldbach-Mansky R, Reiff A, Kastner DL. Mutational analysis in neonatal-onset multisystem inflammatory disease: comment on the articles by Frenkel et al and Saito et al [letter]. Arthritis Rheum 2006;54:2703–4.
5. Frenkel J, van Kempen MJ, Kuis W, van Amstel HK. Variant chronic infantile neurologic, cutaneous, articular syndrome due to a mutation within the leucine-rich repeat domain of CIAS1 [letter]. Arthritis Rheum 2004;50:2719–20.
6. Kanazawa N, Okafuji I, Kambe N, Nishikomori R, Nakata-Hizume M, Nagai S, et al. Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-κB activation: common genetic etiology with Blau syndrome. Blood 2005;105:1195–7.
7. Nakamura Y, Kambe N, Saito M, Nishikomori R, Kim YG, Murakami M, et al. Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. J Exp Med 2009;206:1037–46.

DOI 10.1002/art.27618

Reply

To the Editor:

We thank Dr. Kambe and colleagues for their comments. As mentioned by Kambe et al and in our article, research on the function of NLRP3 in NF-κB signaling has led to many conflicting data. Several studies have shown an activating effect of NLRP3 in the presence of ASC (1–5), while

Modeling Retinal Degeneration Using Patient-Specific Induced Pluripotent Stem Cells

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Abstract

Retinitis pigmentosa (RP) is the most common inherited human eye disease resulting in night blindness and visual defects. It is well known that the disease is caused by rod photoreceptor degeneration; however, it remains incurable, due to the unavailability of disease-specific human photoreceptor cells for use in mechanistic studies and drug screening. We obtained fibroblast cells from five RP patients with distinct mutations in the *RP1*, *RP9*, *PRPH2* or *RHO* gene, and generated patient-specific induced pluripotent stem (iPS) cells by ectopic expression of four key reprogramming factors. We differentiated the iPS cells into rod photoreceptor cells, which had been lost in the patients, and found that they exhibited suitable immunocytochemical features and electrophysiological properties. Interestingly, the number of the patient-derived rod cells with distinct mutations decreased *in vitro*; cells derived from patients with a specific mutation expressed markers for oxidation or endoplasmic reticulum stress, and exhibited different responses to vitamin E than had been observed in clinical trials. Overall, patient-derived rod cells recapitulated the disease phenotype and expressed markers of cellular stresses. Our results demonstrate that the use of patient-derived iPS cells will help to elucidate the pathogenic mechanisms caused by genetic mutations in RP.

Citation: Jin Z-B, Okamoto S, Osakada F, Homma K, Assawachananont J, et al. (2011) Modeling Retinal Degeneration Using Patient-Specific Induced Pluripotent Stem Cells. PLoS ONE 6(2): e17084. doi:10.1371/journal.pone.0017084

Editor: Mark Mattson, National Institute on Aging Intramural Research Program, United States of America

Received October 28, 2010; **Accepted** January 15, 2011; **Published** February 10, 2011

Funding: This study was supported by the grant from the Ministry of Health, Labour and Welfare, Japan (#H21-Nanchi-Ippan-216). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Retinitis pigmentosa (RP) leads inevitably to visual impairment due to irreversible retinal degeneration, specifically of primary rod photoreceptors. The condition causes night blindness and visual field defects. The disease onset spans a wide range of ages, but RP most often occurs in late life. There is no treatment that allows patients to avoid deterioration of visual function. RP encompasses a number of genetic subtypes, with more than 45 causative genes and a large number of mutations identified thus far. The genetic heterogeneity of RP suggests a diversity of disease mechanisms, which remain largely unclear. Furthermore, for many of the RP subtypes, no appropriate animal models are available. Although large clinical trials have been conducted with α-tocopherol and β-carotene, these studies found no statistically significant change of visual function in RP patients [1,2]. The underlying mutations causing disease in the patients tested in the clinical trials were not revealed, and the variability of individual responses to these drugs is unknown. One of the reasons why these clinical trials failed to examine the effectiveness of drugs is that the effect of a drug may be different between patients with different underlying mutations.

Induced pluripotent stem (iPS) cells reprogrammed from somatic cells [3,4] have enabled us to easily generate patient-derived terminally differentiated cells *in vitro* [5–7]. We have successfully induced differentiation of photoreceptor cells from both human embryonic stem (ES) cells [8] and iPS cells [9,10]. Modeling pathogenesis and treatment *in vitro* using patient iPS cell-derived photoreceptors will elucidate disease mechanisms; circumvent problems related to differences among species that arise when using animal models; decrease patient risk; and reduce the cost of early-stage clinical trials. Here, we generated iPS cells from RP patients with different mutations and demonstrated the potential of patient-derived photoreceptors for disease modeling.

Materials and Methods

RP patients and genetic mutations

The protocol of this study adhered to the tenets of the Declaration of Helsinki. The study was approved by the ethical committees of the Institute of Biomedical Research and Innovation Hospital and the RIKEN Center for Developmental Biology, Japan. Written informed consent from all patients was obtained. We selected five RP patients from four families whose disease-causing mutations have been identified (**Fig. 1A–D and Fig. S1**). Of the five RP patients in this study, three late-onset patients carried the following mutations: 721Lfs722X in *RP1*, W316G in *PRPH2*, and G188R in *RHO*. Two relatively early-onset patients from the same family carried a H137L mutation in *RP9*, which we

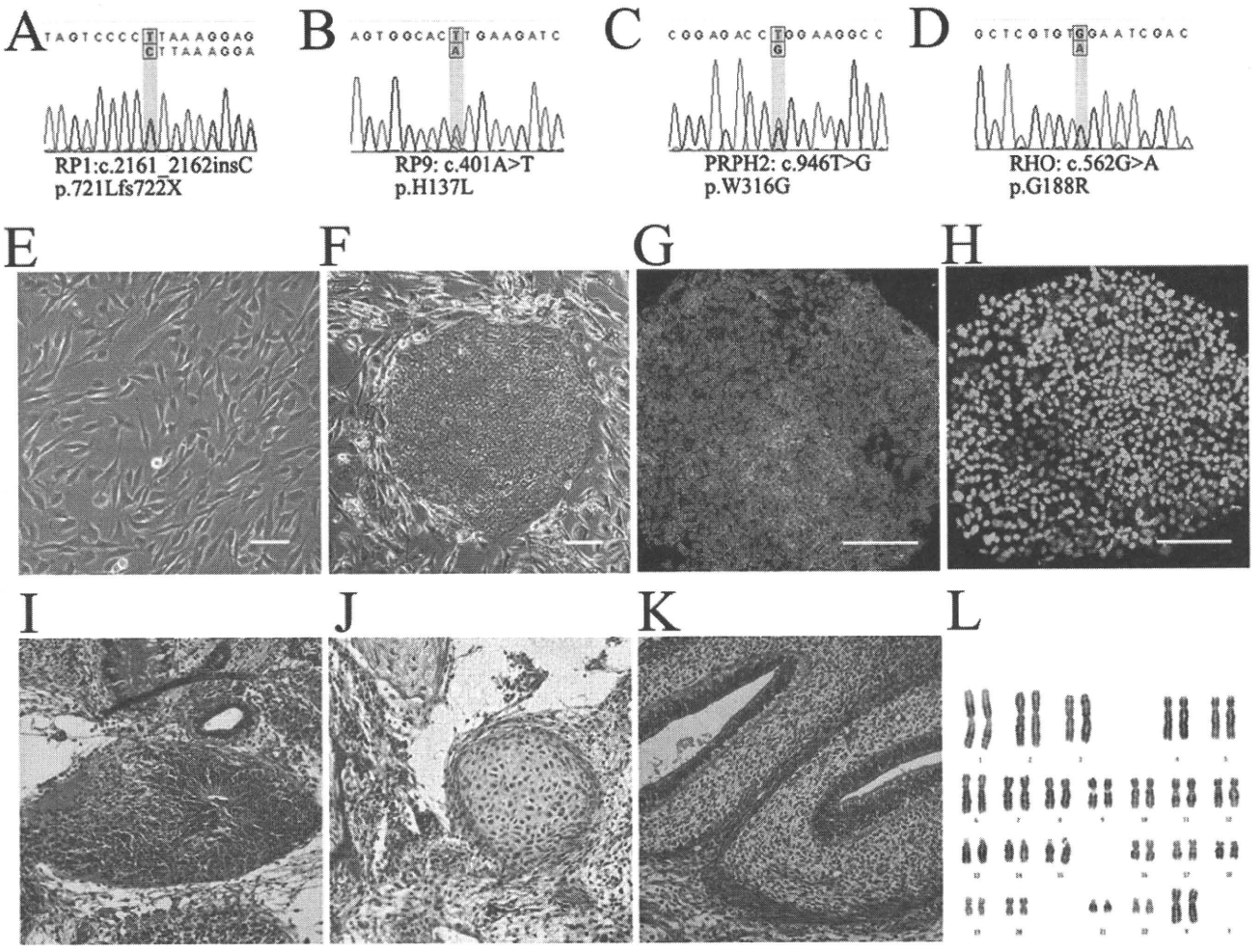

Figure 1. iPS cells derived from RP patients. Mutations identified in patients K21 (RP1) (**A**), K11 and K10 (RP9) (**B**), P101 (PRPH2) (**C**), and P59 (RHO) (**D**). Patient-derived fibroblast cells (**E**) were reprogrammed into iPS cells (**F**). The iPS cells expressed SSEA-4 (**G**) and Nanog (**H**). A teratoma formation test confirmed iPS cells' ability to generate all three germ layers: endoderm (**I**), mesoderm (**J**) and ectoderm (**K**). Karyotype analysis (**I**). Scale bars, 50 μm.
doi:10.1371/journal.pone.0017084.g001

confirmed by both genomic and cDNA sequencing (**Fig. S2**). All patients showed typical manifestations of RP (**Tab. S1**). Peripheral blood obtained from patients was used for DNA isolation. A comprehensive screening of disease-causing genes was carried out as described previously [11]. For the RP9 mutation, total RNA was isolated from fresh blood samples and iPS cells, and synthesized cDNA was subjected to PCR and direct sequencing to confirm whether the mutation was located in the *RP9* gene or the pseudo-RP9 gene (paralogous variant). Both fibroblast and iPS cells were analyzed to re-confirm the identified mutation.

iPS cells generation

To generate iPS cells, retroviral transduction of Oct3/4, Sox2, Klf4, and c-Myc into patient-derived fibroblast cells was carried out as described previously [3]. Established iPS cell lines were maintained on a feeder layer of mitomycin C-treated SNL cells (a murine-derived fibroblast STO cell line expressing the neomycin-resistance gene cassette and LIF) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were maintained in DMEM-F12 supplemented with 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 20% KnockOut Serum Replacement (KSR), and 4 ng/ml basic fibroblast growth factor (Upstate Biotechnology).

Transgene quantification

To examine the copy number of transgenes integrated into the host genome, DNA was isolated and quantitative detection of viral transgenes was performed using real-time PCR. The endogenous gene was used as a control. Before quantitative PCR, a standard curve for each primer and/or probe set was determined using a set of plasmid DNA dilutions. Taqman qPCR to detect integrated OCT3/4, KLF4, and MYC was performed using 20 μl reactions consisting of 10 μl TaqMan Master Mix with uracil N-glycosylase, 4.9 μM primers, 250 nM probe, and 1 μl of the DNA sample. Quantification of viral SOX2 was assayed using SYBR Green.

Teratoma formation

Animal protocols were approved by the RIKEN Center for Developmental Biology ethical committee (No. AH18-05). A total of 10^7 trypsinized iPS cells were injected subcapsularly into the testis of SCID mice (two mice per iPS cell line). Four weeks later, the testis was fixed and sectioned for H&E staining.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min at 4°C and then permeabilized with 0.3% Triton X-100 for 45 min. After 1 h blocking with 5% goat serum, cells were incubated with primary antibodies overnight at 4°C and subsequently with secondary antibodies for 1 h at room temperature. The primary and second antibodies used are listed in **Tab. S2**.

Karyotype analysis

Karyotype analysis of the iPS cell chromosomes was carried out using a standard G-band technique (300–400 band level).

Photoreceptor differentiation and drug testing

In vitro differentiation of rod photoreceptor cells was performed as previously reported [8], but with a minor modification. To find a KSR optimal for retinal differentiation, lot testing was conducted before differentiation. iPS colonies were dissociated into clumps with 0.25% trypsin and 0.1 mg/ml collagenase IV in PBS containing 1 mM $CaCl_2$ and 20% KSR. Feeder cells were removed by incubation of the iPS cell suspension on a gelatin-coated dish for 1 h. iPS clumps were moved to a non-adhesive MPC-treated dish (NUNC) in maintenance medium for 3 days, in 20% KSR-containing differentiation medium (DMEM-12 supplemented with 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine) for 3 days, then in 15% KSR-containing differentiation medium for 9 days, and finally in 10% KSR-containing medium for 6 days. Cells were treated with Lefty-A and Dkk-1 during floating culture. At day 21, the cells were plated en bloc on poly-D-lysine/laminin/fibronectin–coated 8-well culture slides (BD Biocoat) at a density of 15–20 aggregates/cm^2. The cells were cultured in 10% KSR-containing differentiation medium until day 60. Cells were further treated with 100 nM retinoic acid (Sigma) and 100 μM taurine (Sigma) in photoreceptor differentiation medium (GMEM, 5% KSR, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM pyruvate, N2 supplement, and 50 units/ml penicillin, 20 μg/ml streptomycin). Differentiated cells from both normal and patient iPS cells were treated with 100 μM α-tocopherol, 200 μM ascorbic acid and 1.6 μM β-carotene starting at differentiation day 120. One week later, cells were fixed for immunostaining.

Electrophysiological recording

Recombinant lentiviral vectors expressing GFP under the control of the Nrl or RHO promoters were generated in HEK293t cells (RIKEN Cell Bank), and differentiated cells were infected with virus on day 90. Cells expressing GFP were targeted for patch clamp recordings. Voltage-clamp recordings were performed with 12–15 MΩ glass electrodes. Signals were amplified using Multiclamp 700B amplifiers (Molecular Devices). The internal solution was 135 mM K-gluconate, 10 mM HEPES, 3 mM KCl, 0.2 mM EGTA, 2.5 mM $MgCl_2$, 5 mM adenosine 5′-triphosphate, 0.3 mM guanosine-5′-triphosphate, 0.06 mM Alexa Fluor 594 (Molecular probes), adjusted to pH 7.6 with KOH. The retinal cells were perfused with oxygen-bubbled external medium: 23 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 120 mM NaCl, 3.1 mM KCl, 6 mM Glucose, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 0.004% Phenol red. The medium was heated to 37°C with a temperature controller (Warner Instruments).

Cell count and statistical analysis

Differentiated cells visualized with specific antibodies were counted blindly by an independent observer. Data are expressed as means ± s.e.m. The statistical significance of differences was determined by one-way ANOVA followed by Tukey's test or Dunnett's test, or by two-way ANOVA followed by Bonferroni test using the GraphPad Prizm software. Probability values less than 0.05 were considered significant.

Results

Generation of iPS cell lines from patients with RP

Mutations identified in the five patients were confirmed by bi-directional sequencing (**Fig. S1**). Through genotyping of four patients and two normal relatives in the RP9 family, we found the H137L mutation in the *RP9* gene co-segregated with the disease, strongly indicating that the mutation is indeed the genetic cause of the disease. We cultured fibroblasts from skin samples of these patients on gelatin-coated dishes (**Fig. 1E**) and infected them with retroviral vectors encoding *OCT3/4* (also known as *POU5F1*), *SOX2*, *KLF4*, and *c-MYC*, using a previously established method [3]. Each mutation was re-confirmed in both fibroblasts and iPS cells. Established iPS colonies showed human embryonic stem cell-like morphology (**Fig. 1F and Fig. S3A**) and expressed pluripotency markers (**Fig. 1C–D**). We selected iPS cell lines for each patient using multiple criteria. First, we excluded iPS cell lines in which spontaneous differentiation occurred repeatedly during maintenance (**Fig. S3B**). We chose iPS colonies that maintained morphologies similar to those of human ES cells through more than 10 passages. Second, we quantified the transgene copy number and selected iPS cell lines with the fewest integrations, as the risk of gene disruption through random insertion increases with the number of transgenes (**Fig. S4A–E**). Third, in order to select iPS cell lines with full pluripotency, we verified the ability to form teratomas. Teratomas formed by injecting iPS colonies into the testis *in vivo* showed contributions to all three embryonic germ layers: ectoderm, mesoderm, and endoderm (**Fig. 1E–G**). Finally, karyotype analysis was carried out to examine the chromosome integrity. The patient-iPS cells showed normal karyotypes after extended passage, indicating chromosomal stability (**Fig. 1H**). These results provide *in vitro* and *in vivo* functional proof of pluripotency for RP patient-derived iPS cells.

Generation of patient-specific retinal photoreceptor

We previously demonstrated *in vitro* differentiation of retinal photoreceptor cells from wild-type human ES [8] and iPS cells [9,10] using a stepwise differentiation method known as serum-free culture of embryoid body-like aggregates [12]. We first evaluated the differentiation efficiency of three selected iPS cell lines of the five patients (**Fig. 2A**). Retinal progenitor, photoreceptor precursor, retinal pigment epithelium (RPE) and rod photoreceptor cells were sequentially induced (**Fig. 2B–K**), consistent with our previous studies [8–10,12]. All patient-derived iPS cell lines differentiated into RPE cells that form ZO-1+ tight junctions on differentiation day 60, with timing, morphology, and efficiency similar to that of wild-type iPS cells (**Fig. 2D–E; Fig. S5**). Immature photoreceptors expressing Crx and Recoverin (day ~60) were observed as clusters in the colonies (**Fig. S6A–B**). The patient-iPS cells also differentiated into blue Opsin+ or red/green Opsin+ cone photoreceptor cells (**Fig. 2H** and data not shown). Immunostaining of Rhodopsin (a marker of mature rod photoreceptors) revealed no Rhodopsin+ cells at differentiation day 100 (data not shown). Rhodopsin+ cells appeared at differentiation day 120 with a stable efficiency of the three independent iPS cell lines from each patient (**Fig. 2K,N and Fig. S6C**). Additionally, 15.1±0.60% and 13.3±1.65% cells were positive for Recoverin (a conventional marker for both rod, cone photoreceptors and cone bipolar cells) in K21- and K11-iPS cells, respectively

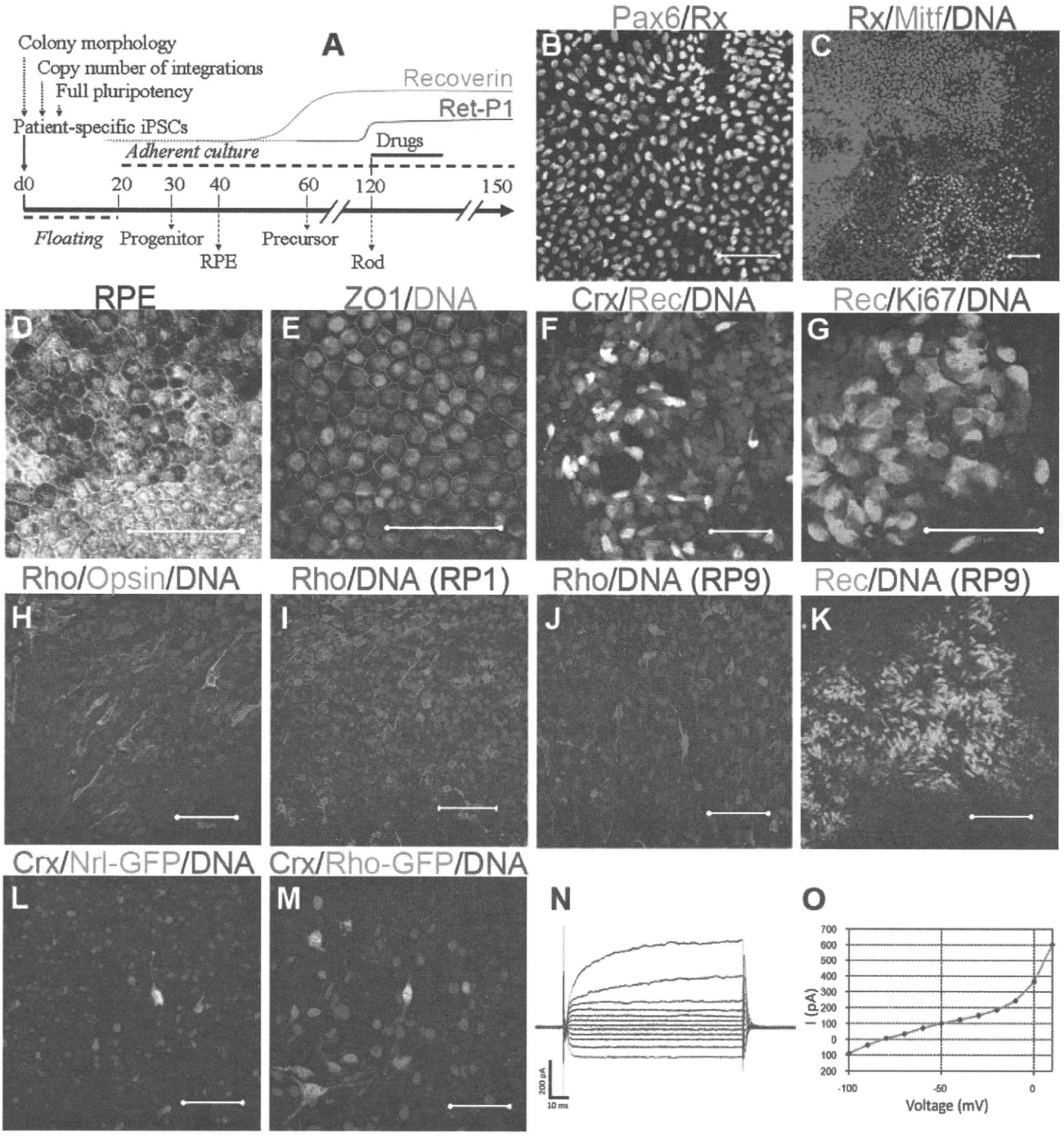

Figure 2. Induction of patient-specific retinal photoreceptor cells. Retinal cells were induced sequentially by *in vitro* differentiation. (**A**) Experimental schema. (**B**) Neural retina progenitor cells (Pax6+Rx+) and RPE progenitor cells (Mitf+) were separated in the culture dish (**C**). Patient-specific RPE cells exhibited hexagonal morphology and pigmentation (**D**) and expressed the tight junction marker ZO-1 (**E**). Photoreceptor cells were positive for immature photoreceptor markers Crx and Recoverin on day 60 (**F**). Recoverin+ cells did not co-express Ki67, a proliferating cell marker (**G**). Differentiation of rod photoreceptors (Rhodopsin+) and cone photoreceptors (Opsin+) from patient iPS cells (**H**). Rhodopsin + rod photoreceptors induced from K21-iPS at day 120 (**I**). K11-derived rod photoreceptors were observed at day 120 (**J**). No Rhodopsin+ cells were detected, but Recoverin+ cells were present at day 150(**K**). Induced rod photoreceptor cells (Crx+) labeled with lentiviral vectors encoding GFP driven by a rod photoreceptor-specific promoter Nrl (**L**: Nrl-GFP) or Rhodopsin (**M**: Rho-GFP). Arrows indicate cells co-expressing Crx and GFP. (**N**) Whole-cell recording of rod photoreceptor cell differentiated human iPS cells. Recorded cells expressed GFP under the control of the Rhodopsin promoter. (**O**) Relationship between voltage and membrane current (i) produced a non-linear curve, suggesting that voltage-dependent channels exist in iPS cell-derived rod photoreceptors Rec, Recoverin; Rho, Rhodopsin. Scale bars, 50 μm.
doi:10.1371/journal.pone.0017084.g002

(data from three selected lines), consistent with stable differentiation. Furthermore, we confirmed rod induction by labeling with lentiviral vectors driving GFP from the Rhodopsin and Nrl promoters, either of which is specifically expressed in rod photoreceptors (**Fig. 2L–M**). Whole-cell patch-clamp recording demonstrated that the rod photoreceptor cell membrane contains voltage-dependent channels, suggesting that differentiated patient-derived rod cells are electrophysiologically functional (**Fig. 2N–O**). Meanwhile, the excluded iPS cell lines (ones that showed spontaneous differentiation during maintenance, or had a high copy number of transgenes), demonstrated a significant diversity of differentiation (**Fig. S7**). Together, these data show that patient-derived iPS cells can differentiate into cells that exhibit many of the immunochemical and electrophysiological features of mature rod photoreceptor cells.

Patient-specific rod cells undergo degeneration *in vitro*

As compared with normal iPS cells, there is no significant difference in rod cell differentiation efficiency at day 120 in K21(RP1)-, P101(PRPH2)-, and P59(RHO)-iPS cell lines (**Fig. 3**). iPS cells from both K11(RP9) and K10(RP9) carried a RP9 mutation; however, rod cell number was significantly lower than in normal iPS cells (**Fig. 3**). We asked whether early death of precursor cells leads to a smaller number of mature rod photoreceptor cells. To determine whether genetic mutations induce degeneration in photoreceptors cells *in vitro*, we extended the culture period and evaluated the number of rod photoreceptors at day 150. In differentiated iPS cells from patient K21(RP1) at day 150, the number of Rhodposin+ cells was significantly decreased (**Fig. 3**). For the K11-iPS cells, no Rhodposin+ cells were found at day 150 (**Fig. 3**). Importantly, some K11-cells at day 150 were positive for Recoverin (10.3±1.99%) and Crx, markers for the rod, cone photoreceptors, and/or bipolar cells (**Fig. 2K** and data not shown), strongly suggesting that cone photoreceptor and/or bipolar cells survived, whereas the rod photoreceptors underwent degeneration *in vitro*. In addition, we detected cells positive for Islet1 (a marker for retinal amacrine, bipolar and ganglion cells), again consistent with the survival of other types of retinal cells (**Fig. S6F**). From these results, we concluded that mature rod photoreceptors differentiated from patient iPS cells selectively degenerate in an RP-specific manner *in vitro*.

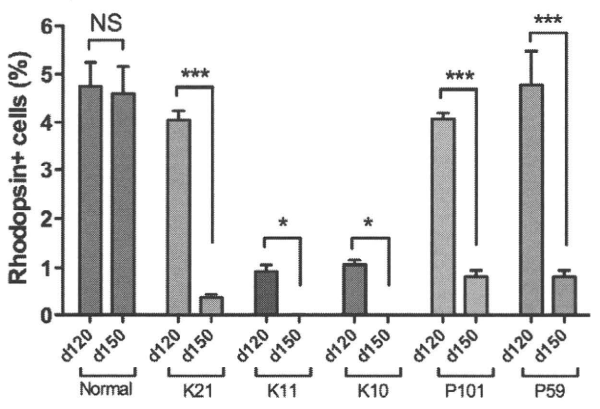

Figure 3. RP patient-derived rod photoreceptors undergo degeneration *in vitro*. iPS cells were differentiated into Rhodopsin+ rod photoreceptors in serum-free culture of embryoid body-like aggregates (SFEB culture). The percentages of Rhodopsin+ rod photoreceptors were evaluated at both day 120 and day 150, respectively. Data were from three independent iPS cell lines derived from the patients. ANOVA followed by Dunnett's test. * $p<0.05$; ***$p<0.001$. Values in the graphs are means and s.e.m.
doi:10.1371/journal.pone.0017084.g003

Cellular stresses involved in patient-derived rod cells

We next asked how the patient-derived rod photoreceptors degenerate. We evaluated apoptosis and cellular stresses in each cell line at both day 100 and day 120, respectively. Interestingly, in the RP9-iPS (K10 and K11) cells, a subset of Recoverin+ cells co-expressed cytoplasmic 8-hydroxy-2'-deoxyguanosine (8-OHdG), a major oxidative stress marker, indicating the presence of DNA oxidation in RP9 patient-derived photoreceptors by differentiation day 100 (**Fig. 4A** and **Fig. S8**). More caspase-3+ cells were presented in the Crx+ photoreceptor cluster of RP9-iPS than in those from other lines (**Fig. 4C–D**). After maturation of the rod photoreceptors from RP9-iPS cells, Rhodopsin+ cells co-expressed Acrolein, a marker of lipid oxidation (**Fig. 4E**), while no Rhodopsin+/Acrolein+ cells were observed in iPS cells derived from other patients carrying different mutations or in normal iPS cells (**Fig. 4F**). This pattern was similar to the cases of 8-OHdG and activated caspase-3. Thus, we conclude that oxidation is involved in the RP9-rod photoreceptor degeneration.

In differentiated RHO-iPS (P59) cells, we found that Rhodopsin proteins were localized in the cytoplasm (**Fig. 4G**), as determined by immunostaining with anti-Rhodopsin antibody (Ret-P1). This pattern is unlike the normal localization of Rhodopsin at the cell membrane in photoreceptors derived from normal iPS or other patient-derived iPS cells (**Fig. 4H** and data not shown). This result suggests accumulation of unfolded Rhodopsin, as reported previously in rhodopsin mutant mice cells [13]. We next examined the possible involvement of endoplasmic reticulum (ER) stress in RHO-iPS cell line degeneration. The Rhodopsin+ or Recoverin+ cells co-expressed immunoglobulin heavy-chain binding protein (BiP) or C/EBP homologous protein (CHOP), two conventional markers of endoplasmic reticulum (ER) stress, from day 120 (**Fig. 4I,K and Fig. S9**), while cells derived from control iPS or other mutant iPS cells were negative for BiP and CHOP (**Fig. 4J,L**). Taken together, these results demonstrate that ER stress is involved in rod photoreceptors carrying a RHO mutation.

Drug evaluation in patient-specific rod cells

The antioxidant vitamins α-tocopherol, ascorbic acid, and β-carotene have been tested in clinical trials as dietary therapies for RP [2] and in another major retinal degenerative disease, age-related macular degeneration [14]. Thus far, mostly due to the lack of appropriate validation models, there has been no evidence supporting the beneficial effects of these compounds on rod photoreceptors. We therefore assessed the effects of these agents on rod photoreceptors derived from patient iPS cells. In mouse retinal culture, short-term treatment with α-tocopherol, ascorbic acid and β-carotene at 100 μM, 200 μM and 1.6 μM, respectively, exerted no significant toxic effects on rod photoreceptor cells (**Fig. S10**). Since the differentiated rod photoreceptors underwent degeneration after day 120, we treated the cells for 7 days with these agents starting at day 120 (**Fig. 2A**). α-Tocopherol treatment significantly increased the number of Rhodopsin+ cells in iPS cells derived from K11- and K10-iPS with the RP9 mutation, while it had no significant effects on iPS cells with the either the RP1, PRPH2 or RHO mutation (**Fig. 5**). In contrast, neither ascorbic acid nor β-carotene treatment had any effect on iPS cells of any genotype (**Fig. S11**). We cannot currently explain the discrepancy between the effects of these antioxidants. It has been reported that under certain circumstances, anti-oxidants can act as "pro-oxidants" [15]. Taken together, our results indicate that treatment with α-tocopherol is beneficial to RP9-rod photoreceptor survival, and causes different effects on Rhodposin+ cells derived from different patients.

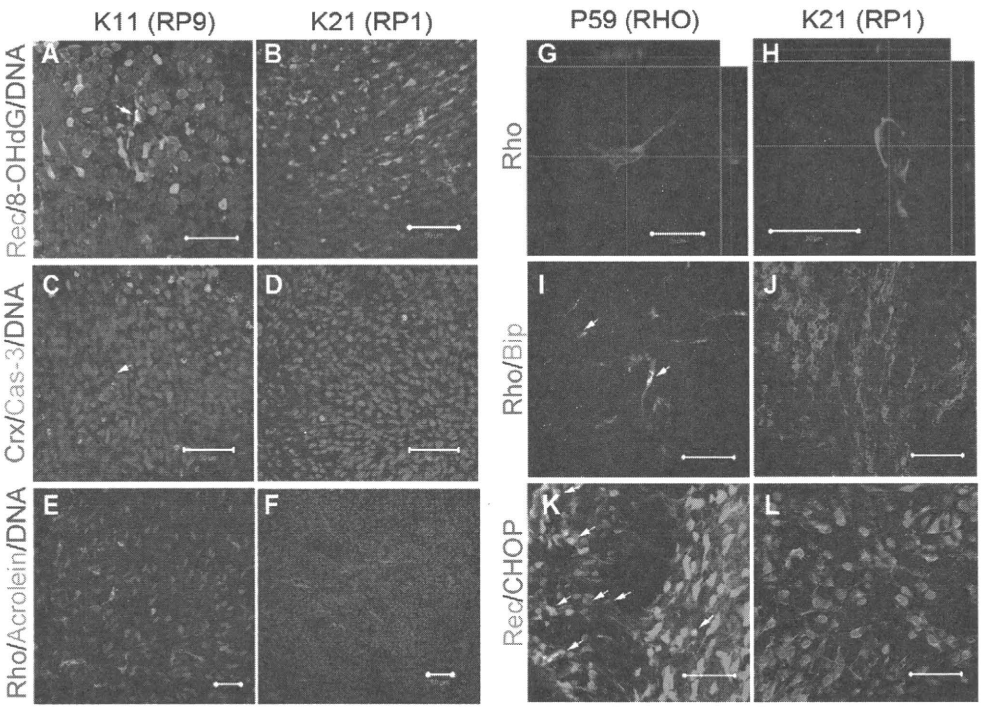

Figure 4. Cellular stress in patient-derived rod photoreceptor cells. Oxidative stress and apoptosis in differentiated rod photoreceptor cells derived from RP9-iPS (**A,C,E**) and RP1-iPS (**B,D,F**). (**A**) 8-OHdG, a marker for DNA oxidation, was found in K11- or K10-iPS–derived differentiated cells (day 100), but not in K21-iPS (**B**). Arrow indicates a cell double-positive for 8-OHdG and Recoverin. (**C**) The number of activated Caspase-3+ cells was greater in K11-iPS differentiation than in K21-iPS (**D**). From day 120, rod photoreceptor cells (Rhodopsin+) derived from RP9-iPS co-expressed the oxidative stress marker Acrolein (**E**); whereas RP1-iPS derivatives did not (**F**). (**G–L**) Abnormal cellular localization of Rhodopsin proteins and endoplasmic reticulum stress in RHO-iPS–derived rod photoreceptors. High magnification revealed cytoplasmic localization of Rhodopsin in rod photoreceptor cells carrying a RHO mutation (**G**) and a normal localization in the cell membrane in K21 cells (**H**). Rod cells derived from RHO-iPS co-expressed the ER stress markers BiP (**I**) and CHOP (**K**). K21-iPS–derived rod cells did not express BiP (**J**) or CHOP (**L**). Arrows indicate double-positive cells. Rec, Recoverin; Rho, Rhodopsin. All scale bars are 50 μm except for G and H (20 μm).
doi:10.1371/journal.pone.0017084.g004

Discussion

By using patient-derived iPS cells and *in vitro* differentiation technology, we have shown that RP9-retinitis pigmentosa is involved, at least in part, in oxidative stress pathways; this has not been reported previously in any animals or cell models. Furthermore, we have demonstrated that the antioxidant α-tocopherol exerts a beneficial effect on RP9-rod cells. Additionally, we have clearly shown that rod photoreceptors derived from patients with a RHO mutation are associated with ER stress; this is

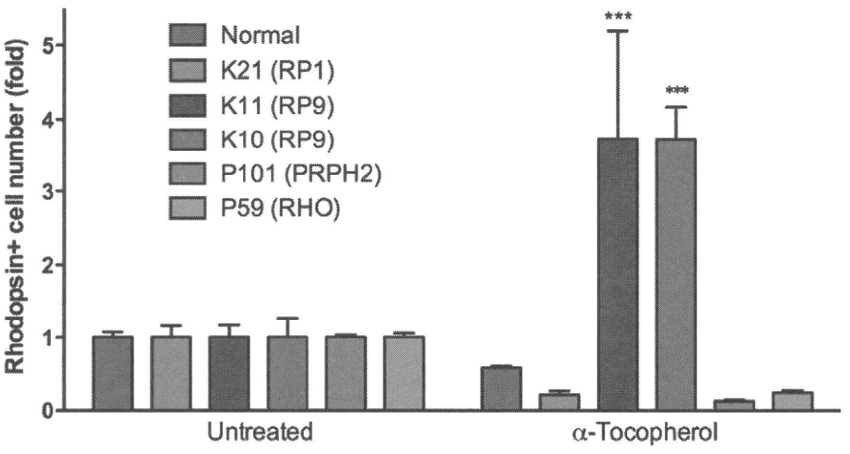

Figure 5. Disease modeling of patient-derived rod photoreceptor cells. α-Tocopherol treatment of patient-specific rod photoreceptors yielded a significant beneficial effect in RP9 mutant cells. Two-way ANOVA Bonferroni post-test showed no significance in other group (n = 3–8). Data represent 1–2 selected iPS cell lines of each patient. ***$p<0.001$. Values in the graphs are means and s.e.m.
doi:10.1371/journal.pone.0017084.g005

the first report of ER stress in a cell culture model for human rod cells. These cell models will be very useful for disease mechanism dissection and drug discovery. By screening several drugs that had already been tested in RP patients, we have revealed that rod photoreceptor cells derived from RP patients with different genetic subtypes exhibit significant differences in drug responses. Among the different types of antioxidants, α-tocopherol has either beneficial or non-beneficial effects on diseased photoreceptors, depending on the genetic mutation. This is the first report of the utilization of iPS cells related to personalized medicine, which will be helpful for routin clinical practice. Our results also provided evidence that genetic diagnosis is essential for optimizing personalized treatment for patients with retinal degenerative diseases [11]. An important future study made possible by this work is the screening of a compound library for drugs that could be used to treat RP. Patient-derived iPS cells revealed differences in pathogenesis and the efficacy of antioxidants among patients with different disease-causing mutations. Although the microenvironment affects the pathogenesis of diseases, and *in vitro* evaluation is not perfect, this study suggests that iPS cells could be used to select between multiple available treatments, allowing physicians to advise each patient individually. The weakness of our method for disease modeling is that differentiation requires a long period of time. Shortening the induction period and identifying appropriate surface markers for rod cells will improve disease modeling using patient-specific iPS cells.

In brief, we generated pluripotent stem cells from retinitis pigmentosa patients and induced them into retinal cells. Compared with normal cells, patient-derived rod cells simulated the disease phenotype and exhibited different responses to specific drugs. We found that patient-specific rod cells underwent degeneration *in vitro*, which maybe related to different cellular stresses. To our knowledge, this is the first report of disease modeling of retinal degeneration using patient-derived iPS cells.

Supporting Information

Figure S1 Pedigrees of K21 (A), P59 (B), K10 and K11 (C). Families of P59 (**B**) and K10 and K11 (**C**) show autosomal dominant mode of inheritance. (**C**) Mutation analysis was performed in four patients and two normal relatives in the RP9 family. The H137L mutation in RP9 gene was co-segregated with the disease in the family. Closed symbols indicate individuals with RP and open symbols indicate unaffected subjects. Question marks indicate symptom unknown. The bars above the symbols indicate examined subjects. Arrow, proband; slash, deceased.
(TIF)

Figure S2 Mutation in the *RP9* gene. (**A**) Alignment of RP9 sequence and pseudo-gene shows the same nucleotide in the mutated location. (**B**) Sequence chromatogram of cDNA sequence demonstrates the c.410A>T (H137L) mutation in the *RP9* gene, instead of the paralogous variant in pseudo-gene which was documented in RetNet (www.sph.uth.tmc.edu/retnet/disease.htm).
(JPG)

Figure S3 Selection by colony morphology. (**A**) iPS colony (K21S4) shows ES-like morphology. (**B**) Spontaneous differentiation in the colony during maintenance (K21S14). Scale bars, 50 μm.
(TIF)

Figure S4 Quantification of transgene copy number. Total copy number of four transgenes in the selected iPS lines. Selected iPS cells with fewest integrations and two high copy number lines used for *in vitro* differentiation.
(TIF)

Figure S5 Efficiency of RPE induction in patient-iPS cells. RPE production of the five patient-iPS cells showed no significant differences (n = 4). Data represent the percentage of RPE area at differentiation day 60. One-way ANOVA followed by Dunnett's test. Values are mean and s.e.m.
(TIF)

Figure S6 Induced retinal cells from patient iPS cells (K21S4). Crx+ photoreceptor precursor cells present in the cell cluster on differentiation day 60 (A). Crx+ cells co-expressed Recoverin, indicating differentiation into photoreceptor cells (B). Rhodopsin+ cells had a long process at day 150 (C). In the differentiated cells, we also observed cells positive of PKCα (a marker for bipolar cells) (D). Cells positive for Math5 and Brn3b (markers for ganglion progenitor or ganglion cells (day 60) (E). Cells positive for Islet-1 (a marker for amacrine, bipolar and ganglion cells) (F). Scale bars, 50 μm (A, D, E, and F); 20 μm (B and C).
(TIF)

Figure S7 Differentiation of the patient-iPS cells. iPS colony was cut into uniform sized pieces (**A**) and subjected to a floating culture (P59M8, day 20) (**B**). RPE (pigmented) and recoverin+ (green) cells were efficiently induced (P59M8, day 60) (**C**). (**D**) An excluded iPS line, P59M16, with high number transgenes showed a striking lentoid formation during the floating culture (day 20). Scale bars, 50 μm.
(TIF)

Figure S8 Oxidative stress in photoreceptor cells with the RP9 mutation (K11). (A) Recoverin, (B) 8-OHdG, (C) Recoverin/8-OHdG, (D) Recoverin/8-OHdG/DNA. Arrows indicate cells with weak Recoverin signal positive for 8-OHdG; Arrowheads represent cells with strong Recoverin signal positive for 8-OHdG; Asterisks represent Recoverin+ cells negative for 8-OHdG. Scale bar, 50 μm.
(JPG)

Figure S9 ER stress in photoreceptor cells with the RHO mutation (P59). (A) CHOP, (B) Recoverin, (C) Recoverin/CHOP, (D) Recoverin/CHOP/DNA. Arrows indicate cells with weak Recoverin signals positive for CHOP in nuclei; Arrowheads represent cells with strong Recoverin signals positive for CHOP; Asterisks represent Recoverin+ cells negative for CHOP. Scale bar, 50 μm.
(JPG)

Figure S10 Toxicity testing of the antioxidants in murine retina-derived rod photoreceptor cells. Primary culture of mouse retinal cells treated with 100 μM α-tocopherol, 200 μM ascorbic acid or 1.6 μM β-carotene for 24 hours and the rod photoreceptors were counted using flow cytometry. Value represents the ratio of treated-rod photoreceptors compared with control cells. n = 4. One-way ANOVA followed by Dunnett's test. Values are mean and s.e.m. NS, not significant.
(JPG)

Figure S11 Differentiated rod cells from normal and patient iPS cells treated with 200 μM ascorbic acid or 1.6 μM β-carotene did not show statistically significant differences. Two-way ANOVA Bonferroni post-test. Values are mean and s.e.m.
(JPG)

Table S1 Phenotypic data of the RP patients. M, male; F, female; AD, age at diagnosis; BCVA, best corrected visual acuity; HM, hand motion.
(DOC)

Table S2 Antibodies used in the present study.
(DOC)

Acknowledgments

We thank C. Ishigami and Y. Tada for assistance of mutation screening; K. Iseki, N. Sakai, Y. Wataoka, K. Sadamoto, A. Tachibana, C. Yamada for technical assistance; Y. Arata, W. Meng, C. Li, A. Suga, M. Mandai and all members in the Takahashi lab for advice.

Author Contributions

Conceived and designed the experiments: ZBJ MT. Performed the experiments: ZBJ SO FO KH JA. Analyzed the data: ZBJ SO FO. Contributed reagents/materials/analysis tools: MT YH TI. Wrote the paper: ZBJ MT.

References

1. Weleber RG, Gregory-Evans K (2006) Retinitis Pigmentosa and Allied Disorders. In: Hilton DR, Schachat AP, Ryan SJ, eds. Retina. Elsevier Mosby. pp 395–498.
2. Berson EL, Rosner B, Sandberg MA, Hayes KC, Nicholson BW, et al. (1993) A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa. Arch Ophthalmol 11: 761–772.
3. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131: 861–872.
4. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917–1920.
5. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, et al. (2008) Disease-specific induced pluripotent stem cells. Cell 134: 877–886.
6. Raya A, Rodríguez-Pizà I, Guenechea G, Vassena R, Navarro S, et al. (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460: 53–59.
7. Yamanaka S (2007) Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell Stem Cell 1: 39–49.
8. Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, et al. (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat Biotechnol 26: 215–224.
9. Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, et al. (2009) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J Cell Sci 122: 3169–3179.
10. Hirami Y, Osakada F, Takahashi K, Okita K, Yamanaka S, et al. (2009) Generation of retinal cells from mouse and human induced pluripotent stem cells. Neurosci Lett 458: 126–131.
11. Jin ZB, Mandai M, Yokota T, Higuchi K, Ohmori K, et al. (2008) Identifying pathogenic genetic background of simplex or multiplex retinitis pigmentosa patients: a large scale mutation screening study. J Med Genet 45: 465–472.
12. Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, et al. (2005) Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci U S A 102: 11331–11336.
13. Sung CH, Davenport CM, Nathans J (1993) Rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa. Clustering of functional classes along the polypeptide chain. J Biol Chem 268: 26645–26649.
14. van Leeuwen R, Boekhoorn S, Vingerling JR, Witteman JC, Klaver CC, et al. (2005) Dietary intake of antioxidants and risk of age-related macular degeneration. JAMA 294: 3101–3107.
15. van Helden YG, Keijer J, Heil SG, Picó C, Palou A, et al. (2009) Beta-carotene affects oxidative stress-related DNA damage in lung epithelial cells and in ferret lung. Carcinogenesis 30: 2070–2076.