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G：知的財産権の出願・登録状況

該当なし。

Ⅳ. 班会議・合同研究カンファレンス

平成22年度厚生労働科学研究費補助金
「新たな移植細胞療法に向けた造血幹細胞の ex vivo 増幅技術の開発と応用」班会議 議事録

第1回合同研究カンファレンス

1．日時　　平成22年　5月　11日（火）　１４：００～１６：００
2．場所　　先端医療センター　臨床棟4階　研修室
3．データカンファレンス

演者	演題名
伊藤　仁也 先端医療センター 細胞管理室　室長	・Ex vivo 増幅臍帯血移植前臨床試験」臨床プロトコル案について
丸山　京子 先端医療センター 細胞管理室　技術研究員 田中　宏和 大阪大学大学院医学系研究科 血液・腫瘍内科	・完全無血清培地を用いた新培養法による実製造試験結果

第2回合同研究カンファレンス

1．日時　　平成22年　12月　21日（火）　１４：００～１６：００
2．場所　　メルパルク京都　6階　会議室3
3．データカンファレンス

演者	演題名
伊藤　仁也 先端医療センター 細胞管理室　室長	・Ex vivo 増幅臍帯血移植臨床例報告（どうして拒絶が起きたのか。） ・新たな移植細胞療法に向けた造血幹細胞の ex vivo 増幅技術の開発と応用（進捗状況）
清水　則夫 東京医科歯科大学 ウイルス治癒学　准教授	「可溶性 Notch リカンド Delta1-Fc を用いて増幅した臍帯血造血幹・前駆細胞移植の臨床研究に向けて」

Ⅴ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表(英文)

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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Kurita N, Nishikii H, Nakamoto R, Nakamura N, Kondo Y, Okoshi Y, Suzukawa K, Hasegawa Y, Yokoyama Y, Noguchi M, Chiba S.	A highly therapy-resistant case with "B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma" showing strong BCL2 staining, otherwise indistinguishable from Burkitt lymphoma.	J Exp Hematol			im press
akata-Yanagimoto M, Sakai T, Miyake Y, Saito TI, Maruyama H, Morishita Y, Nakagami-Yamaguchi E, Kumano K, Yagita H, Fukayama M, Ogawa S, Kurokawa M, Yasutomo K, Chiba S.	Notch2 signaling is required for proper mast cell distribution and mucosal immunity in the intestine.	Blood	117	128-134	2011
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研究成果の刊行に関する一覧表(和文)

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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ORIGINAL ARTICLE

Prognostic significance of the *BAALC* isoform pattern and *CEBPA* mutations in pediatric acute myeloid leukemia with normal karyotype: a study by the Japanese Childhood AML Cooperative Study Group

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Abstract High *BAALC* (brain and acute leukemia, cytoplasmic) gene expression may indicate an adverse prognosis for adults who have acute myeloid leukemia (AML) and a normal karyotype, but its prognostic significance for pediatric AML cases is unclear. Whether different *BAALC* isoform patterns are of prognostic significance is also unclear. Newly diagnosed AML patients with normal karyotype who were treated by the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed in terms of their *BAALC* expression levels ($n = 29$), *BAALC* isoforms ($n = 29$), and *CEBPA* mutations ($n = 49$). Eleven and 18 patients exhibited high and low *BAALC* expression, respectively, but these groups did not differ significantly in terms of overall survival (54.6 vs. 61.1%, $P = 0.55$) or event-free survival (61.4 vs. 50.0%, $P = 0.82$). Three of these 29 patients (10.3%) expressed the exon 1-5-6-8 *BAALC* isoform along with the expected 1-6-8 isoform and had adverse clinical outcomes. Novel

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CEBPA mutations were also identified in four of 49 patients (8.2%). All four patients have maintained complete remission for at least 5 years. Thus, 1-5-6-8 isoform expression may be associated with an adverse prognosis in pediatric AML with normal karyotype. *CEBPA* mutations may indicate a favorable prognosis.

Keywords Pediatric AML · Normal karyotype · *BAALC* · *CEBPA*

1 Introduction

Cytogenetic abnormalities in acute myeloid leukemia (AML) that are detected at the time of diagnosis are important prognostic factors that help to determine the clinical outcome. However, 10–20% of pediatric AML cases lack known genetic abnormalities that can be used to predict clinical outcome [1]. For example, while tandem duplications of mixed lineage leukemia gene (*MLL*) and fms-like tyrosine kinase-3 (*FLT3*) correlate with a poor prognosis in pediatric AML [2], internal tandem duplications (ITD) of *FLT3* occur much more rarely in pediatric AML than in adult AML patients; indeed, there is an age-associated increase in this mutation (from 1.5% in infants to nearly 20% in teenage patients) [3]. Thus, most normal karyotype pediatric AML patients lack known markers that are of prognostic significance. To improve the prognostic stratification of this heterogeneous group of patients, novel markers should be identified.

The *BAALC* (brain and acute leukemia, cytoplasmic) gene is believed to participate in the development of AML and chronic myelogenous leukemia in blast crisis [5]. Previous studies have also reported that high *BAALC* expression levels reflect an adverse prognosis for adult AML with a normal karyotype [6–10]. However, how *BAALC* expression levels relate to the clinical outcome of pediatric AML remains unclear.

CEBPA is a transcription factor that coordinates the granulocytic differentiation of common myeloid progenitors. *CEBPA* mutations have been detected in 7–15% of adult patients with AML and are most frequently found in the AML M1 and M2 subtypes [French–American–British (FAB) classification] [10, 11]. Previous reports indicate that *CEBPA* mutations reflect a favorable prognosis in adult AML with normal karyotype [12, 13]. However, it is unclear whether the same relationship exists between *CEBPA* mutations and pediatric AML.

While previous studies have mainly examined various prognostic factors in terms of gene mutations [9–14] and changes in gene expression [15–20], some recent studies have also reported the prognostic significance of the expression of different isoforms in leukemia [21–25]. Consequently, in the present study, we investigated the prognostic relevance of high *BAALC* expression, *BAALC* isoform patterns, and *CEBPA* mutations in pediatric AML with normal karyotype. This study was performed by the Japanese Childhood AML Cooperative Study Group, which employed the AML 99 protocol [2, 4, 26, 27].

2 Patients and methods

2.1 Patients

This study included 124 of the 241 pediatric patients who were newly diagnosed with de novo AML from January 2000 to December 2002. The 241 patients included 52 patients with a normal karyotype and 49 of those were recruited into the 124-patient group. None of these 49 patients had AML-M3 or Down syndrome. AML was diagnosed according to the FAB classification, and a routine G-banding method was used for cytogenetic analysis. Of the 124 cases, 104 were subjected to *BAALC* expression analysis; of these 104 subjects, 29 had a normal karyotype. These 29 normal karyotype cases were also subjected to *BAALC* isoform analysis. All 49 normal karyotype cases were subjected to *CEBPA* mutation analysis. The characteristics of the patients subjected to *BAALC* isoform analysis and *CEBPA* mutation analysis are shown in Table 1a. The 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses did not differ significantly in age, white blood cell (WBC) counts at diagnosis, remission rates or overall survival (OS) from the remaining 20 normal karyotype patients who were not analyzed for *BAALC* isoform and expression (Table 1b). In accordance with the Declaration of Helsinki and upon approval from the Ethics Committees of Kyoto University, informed consent was obtained from each patient or the patient's parents before entering this study.

2.2 *BAALC* expression analysis

Comparative real-time RT-PCR assays were performed and *BAALC* expression levels were measured as previously reported [5, 6]. The *BAALC* expression values of the patient group were divided at the median value (0.57) and patients were said to have a low and high *BAALC* expression if they had expression values within the lower and upper 50% of values, respectively [7].

2.3 *BAALC* isoform analysis

BAALC isoform analysis was performed by RT-PCR followed by direct sequencing. For this, the PCR product was cut out of the gel, purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and sequenced by the

Table 1 Characteristics of AML patients subjected to *BAALC* expression, *BAALC* isoform, and *CEBPA* mutation analyses

	BAALC		*CEBPA*
	All ($n = 104$)	Normal karyotype ($n = 29$)	Normal karyotype ($n = 49$)
(a)			
FAB			
M0	4	2	4
M1	18	2	6
M2	36	9	12
M4	18	8	12
M5	19	7	12
M7	7	1	3
UN	2	0	0
Age	4 days to 15 years	3 months to 15 years	3 months to 15 years
Sex			
Male	60	16	27
Female	44	13	22
Risk group			
Low	45	0	0
Intermediate	51	29	49
High	8	0	0

	BAALC		*P* value
	Analyzed patients ($n = 29$)	Non-analyzed patients ($n = 20$)	
(b)			
Age			
Median	8 years	8 years	
Range	3 months to 15 years	7 months to 15 years	0.591
WBC at diagnosis ($\times 10^9$/L)			
Median	53.09	15.20	
Range	2.30–343.40	1.20–236.90	0.275
Remission rates	89.7%	100%	0.083
Overall survival	58.6%	65.2%	0.639

UN undifferentiated

dideoxynucleotide termination method with ABI 3100 (Applied Biosystems, Foster City, CA). The primers used are shown in supplementary Table 1. The conditions for the PCR reactions were 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 7 min.

2.4 *CEBPA* mutation analysis

The entire coding region of the gene was amplified using overlapping PCR primer pairs followed by direct sequencing as previously described [28].

2.5 Statistical analysis

Survival distributions were estimated using the Kaplan–Meier method and the differences were compared using the log-rank test. OS and event-free survival (EFS) were defined as the time from diagnosis to death from any cause or the last follow-up and the time from diagnosis to event (relapse or death from any cause), respectively.

3 Results

3.1 *BAALC* expression levels

High *BAALC* expression was associated with M0, M1, and M2 FAB subtypes, while M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). Healthy volunteers (I) have remarkably small range of *BAALC* expression levels compared to AML patients (II, III), as the previous study was reported [15]. We did not observe significant differences between normal karyotype patients

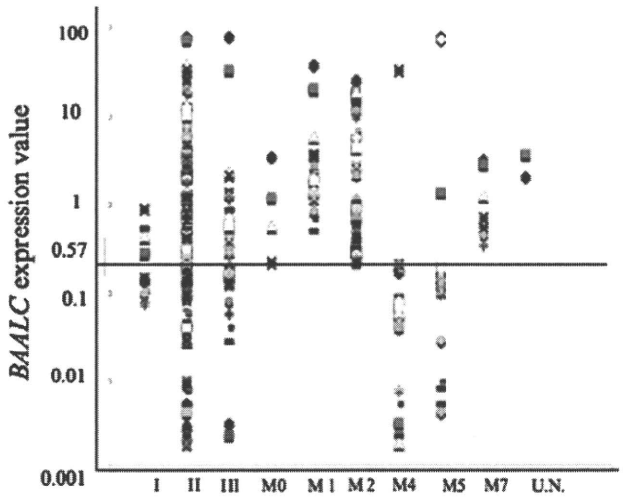

Fig. 1 *BAALC* expression levels in pediatric AML with normal karyotype and FAB subtype patients. The *dot plot* indicates the individual *BAALC* expression levels of healthy volunteers (I, $n = 9$), all AML patients (II, $n = 104$), AML patients with normal karyotype (III, $n = 29$), and the M0 ($n = 4$), M1 ($n = 18$), M2 ($n = 36$), M4 ($n = 18$), M5 ($n = 19$), M7 ($n = 7$), and undifferentiated (U.N., $n = 2$) FAB subtype patients

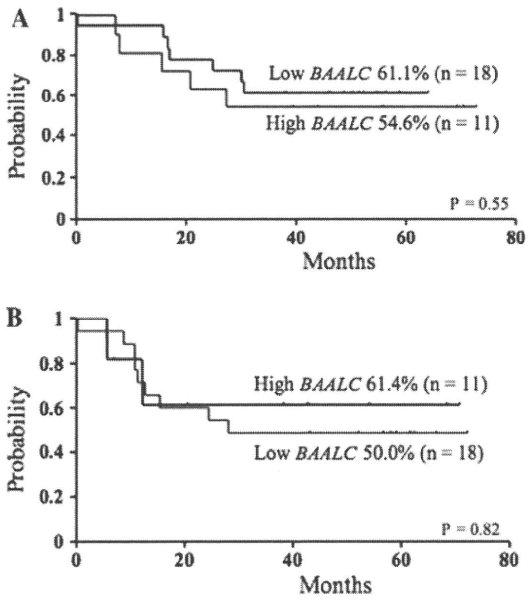

Fig. 2 Kaplan–Meier analysis of the overall survival (OS) and event-free survival (EFS) of pediatric AML patients with normal karyotype who express *BAALC* at high and low levels. The OS (**a**) and EFS (**b**) of the high and low *BAALC*-expressing pediatric AML with normal karyotype patients did not differ significantly

with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b).

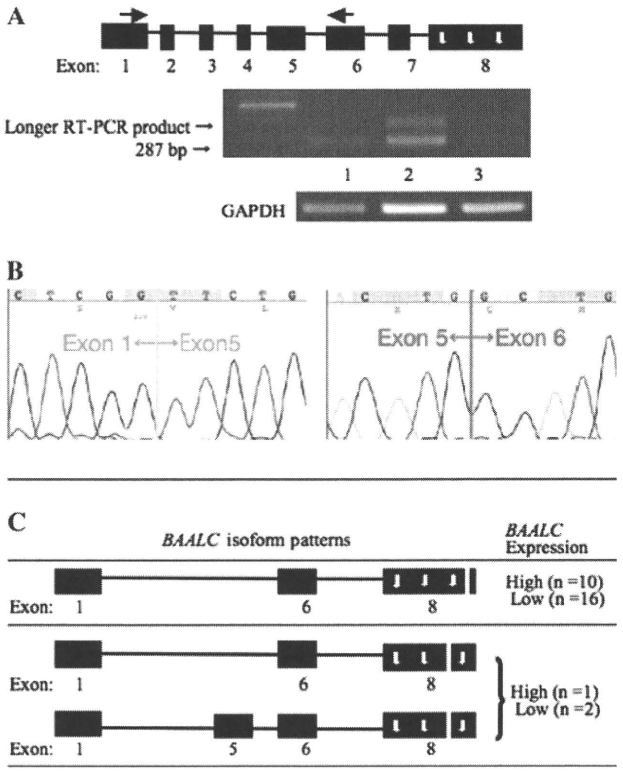

Fig. 3 **a** Schematic depiction of the *BAALC* gene, which consists of eight exons (indicated by *black boxes*). The three polyadenylation signals in the 3′ untranslated region (UTR) of exon 8 that lead to three differently sized transcripts are indicated by *down arrows*. Comparative RT-PCR using primers in exons 1 and 6 revealed that three samples had a longer RT-PCR product in addition to the expected 287 bp product. **b** A partial sequence trace of exons 1, 5, and 6 in the longer RT-PCR product. **c** Schematic depiction of the relationship between *BAALC* isoform patterns and expression levels. Three (10.3%) of the 29 cases had the 1-5-6-8 isoform

3.2 *BAALC* isoform pattern and its relationship to *BAALC* expression levels

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several different transcripts. Five stable isoforms have been detected in leukemic blasts, namely 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. To investigate the prognostic relevance of *BAALC* isoform patterns for pediatric AML patients, we subjected 29 pediatric AML patients with normal karyotype to RT-PCR and direct sequencing. All samples had the predicted product, which consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had a longer RT-PCR product that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). How these isoform patterns relate to the *BAALC* expression levels of the 29 patients is depicted schematically in Fig. 3c. One of three patients with the

Table 2 Characteristics of patients with the 1-5-6-8 *BAALC* isoform

Case no.	*BAALC* expression value	Relapse	Clinical outcome	FAB classification	Age (years)	Sex
1	High (2.43)	+	Dead	M4	13	Female
2	Low (0.39)	−	Dead	M5a	6	Male
3	Low (0.21)	+	Dead	M4	15	Male

Table 3 *CEBPA* mutations

FAB subtype	All ($n = 49$)	*CEBPA* mutations	
M0	4	0	
M1	6	2	c. 1074_1075insAGA
			c. 1092_1093insCAC
M2	12	2	c. 214_224delCCCCGCACGCG
			c. 212_213insC and
			c. 720_726insCGCACC
M4	12	0	
M5	12	0	
M7	3	0	
Total	49	4	

Sequence numbering is according to GenBank accession number U34070

1-5-6-8 isoform had high *BAALC* expression. Of the 26 patients with the 1-6-8 isoform only, 10 and 16 showed high and low *BAALC* expression, respectively. The three patients with the 1-5-6-8 isoform had a poor prognosis (Table 2).

3.3 *CEBPA* mutation

CEBPA mutations were detected in four of the 49 AML with normal karyotype patients (8.2%). Two of these belonged to the M1 subset, and the remaining two belonged to the M2 subset. N-terminal frameshift mutations and in-frame insertions in the basic-leucine zipper (bZIP) domain were detected (Table 3). Novel mutations were identified, namely, c. 212_213insC, c. 214_224delCCCCGCACGCG, c. 720_726insCGCACC, c. 1074_1075insAGA, and c. 1092_1093insCAC. One patient had biallelic mutations in both the N-terminal part and the bZIP domain of *CEBPA*.

4 Discussion

High *BAALC* expression was associated with the M2 subset and the more immature M0/M1 FAB subtypes, while the monocytic-differentiated M4 and M5 FAB subtypes correlated with low *BAALC* expression (Fig. 1). This relationship between *BAALC* expression and FAB subtypes is generally consistent with previously reported observations of adult AML cases [7], although the high *BAALC* expression in the M2 subtype cases was only observed in the pediatric AML patients. *BAALC* expression level was not associated with WBC counts at diagnosis [all AML patients ($n = 104$), $P = 0.91$; AML with normal karyotype ($n = 29$), $P = 0.97$]. The *BAALC* gene is normally expressed by neuroectoderm-derived tissues and CD34-positive hematopoietic progenitor cells and has been implicated in AML and chronic myelogenous leukemia in blast crisis [5]. Recently, quantification of *BAALC* gene expression made it possible to assess MRD in patients with CD34-positive acute leukemia [29]. Little is known about the functions of the *BAALC* gene, but it has been reported to mediate the anabolic action of PTH (parathyroid hormone) on bone cells [30]. It also serves as a marker of the mesodermal lineage (especially muscle) [31] and synaptogenesis [32], and a study on hematopoietic progenitor cells has shown that *BAALC* downregulation occurs upon cell differentiation [33]. Thus, while the functions of the *BAALC* gene remain unclear, our observations are consistent with the notion that it may be associated with monocytic cell differentiation.

We did not observe significant differences between normal karyotype patients with high ($n = 11$) and low ($n = 18$) *BAALC* expression with regard to their OS (54.6 vs. 61.1%, $P = 0.55$, Fig. 2a) or EFS (61.4 vs. 50.0%, $P = 0.82$, Fig. 2b). These results are not consistent with those of a previous study that examined the *BAALC* expression of adult normal karyotype AML patients [7]. In that study, high *BAALC* expression was significantly associated with a poor OS and a higher cumulative incidence of relapse. The discrepancy between this study and ours could reflect the fact that in the other study, the *BAALC* expression values of the patient group were divided at the median value of twelve healthy volunteers, which served as the cutoff [15]. We compared the results according to two different cutoff levels. AML samples were dichotomized at the median value (0.15) of nine healthy volunteers, but we also observed no significant differences of two expression groups (date not shown). To resolve this apparent discrepancy, a larger number of pediatric AML patients will need to be studied.

The *BAALC* gene consists of eight exons and its transcription followed by alternative splicing yields several

different transcripts in leukemic blasts, namely, 1-8, 1-6-8, 1-5-6-8, 1-4-5-6-8, and 1-5-6-7-8 [5]. In our study, all samples of 29 pediatric AML patients with normal karyotype had the *BAALC* isoform that consisted of exon 1, 6, and 8 sequences (1-6-8). However, three samples (10.3%) also had the *BAALC* isoform that contained in addition the exon 5 sequence (1-5-6-8) (Fig. 3a, b). One of the three patients with the 1-5-6-8 isoform also had high *BAALC* expression. With regard to prognosis, all three patients with the 1-5-6-8 *BAALC* isoform have died (Table 2). Two relapsed after complete remission and the third died after intracranial hemorrhage during induction therapy. Of the 29 normal karyotype patients who were subjected to *BAALC* isoform and expression analyses, *FLT3*-ITD were found in eight (27.6%), *FLT3*-D835Mt in two (6.9%), *NRAS* mutations in two (6.9%), and *KRAS* mutations in four (13.8%), but no *NPM1* gene mutations were found [4]. There are no differences of *FLT3*-ITD, *MLL*-PTD and *CEBPA* in high and low *BAALC* expression group (data not shown). Of the three 1-5-6-8 *BAALC* isoform-bearing patients, one had the *FLT3*-ITD mutation and another patient had a *RAS* mutation. A previous study did not detect a difference in outcome between cytogenetically normal adult AML patients with and without *NRAS* mutations [34]. Thus, it seems that the possession of the 1-5-6-8 *BAALC* isoform by pediatric AML patients with normal karyotype may be associated with a candidate for some adverse prognostic factor. Studies with greater patient numbers should be performed to confirm this. Recent reports have suggested that the isoform patterns of other genes (i.e., *AML1-ETO9a* [21], *WT1* [22], *PML/RARa* [23], *Ikaros* [24], and *FHIT* [25]) are of prognostic significance, which supports the significance of investigating the *BAALC* isoform patterns.

CEBPA mutations were detected in four of the 49 AML patients with normal karyotype (8.2%). Two of these belonged to the M1 subset and the remaining two belonged to the M2 subset. One patient had biallelic mutations in both the N-terminal part and bZIP domain of *CEBPA*. To date, two categories of *CEBPA* mutations have been reported: out-of-frame ins/del mutations that often occur in the N-terminal region, and in-frame ins/del mutations that often occur in the C-terminal region [12, 13]. The mutations in both the N-terminal part and bZIP domain have been described in adult AML, but the reported frequencies vary considerably, ranging between 11 and 47% [35]. In a study of pediatric AML patients, of whom six had a normal karyotype, four of the six (67%) had one or more *CEBPA* mutations [36], but the clinical outcomes associated with these mutations are unclear. Notably, in the absence of poor prognostic factors, adult patients with *CEBPA* mutations have been shown to have favorable clinical outcomes [33, 37]. In our study, none of the patients exhibiting a *CEBPA* mutation also had the *FLT3*-ITD mutation and all maintained complete remission for at least 5 years. The statistical significance was not indicated for insufficient sample numbers in AML 99 protocol between normal karyotype patients with CEBPA mutation (+) ($n = 4$) and mutation (−) ($n = 45$) with regard to their OS (100 vs. 55.4%, $P = 0.14$) or EFS (100 vs. 48.9%, $P = 0.09$) (supplementary Fig. 1). But differing from previous report about pediatric AML patients with *CEBPA* mutations, the presentation of clinical information about them may be evaluated. Thus, in the absence of other adverse factors, *CEBPA* mutations may also be suspected to favorable prognostic factors for pediatric AML with normal karyotype.

In summary, we report here for the first time that the presence of the 1-5-6-8 *BAALC* isoform may be associated with a poor prognosis for pediatric AML patients with normal karyotype. In contrast, *CEBPA* mutations are suspected to a good prognosis.

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Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells

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ABSTRACT **Induced pluripotent stem (iPS) cells, which are a type of pluripotent stem cell generated from reprogrammed somatic cells, are expected to have potential for patient-oriented disease investigation, drug screening, toxicity tests, and transplantation therapies. Here, we demonstrated that murine iPS cells have the potential to develop *in vitro* into skeletal muscle stem/progenitor cells, which are almost equivalent to murine embryonic stem cells. Cells with strong *in vitro* myogenic potential effectively were enriched by fluorescence-activated cell sorting using the anti-satellite cell antibody SM/C-2.6. Furthermore, on transplantation into *mdx* mice, SM/C-2.6$^+$ cells exerted sustained myogenic lineage differentiation in injured muscles, while providing long-lived muscle stem cell support. Our data suggest that iPS cells have the potential to be used in clinical treatment of muscular dystrophies.—Mizuno, Y., Chang, H., Umeda, K., Niwa, A., Iwasa, T., Awaya, T., Fukada, S., Yamamoto, H., Yamanaka, S., Nakahata, T., Heike, T. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J.* 24, 2245–2253 (2010). www.fasebj.org**

Key Words: Duchenne muscular dystrophy · Pax7 · long-term engraftment · no teratoma formation · high engraftment efficiency

TO MAINTAIN HOMEOSTASIS, SKELETAL muscle fibers are continuously regenerated by activated satellite cells (1), the muscle-specific stem cells that differentiate into myoblasts and form myotubes to replace the myofibers damaged by exercise and daily activities (2). The muscular dystrophies are inherited myogenic disorders of variable distribution and severity that are characterized by progressive muscle wasting and weakness (3). In many forms of muscular dystrophy, the common molecular defect of the encoded proteins, which are involved in muscular structural integrity, is observed in both immature satellite cells and mature myofibers (4). Duchenne muscular dystrophy (DMD), which is the best-described and most serious form of muscular dystrophy, results from mutations in the X-linked dystrophin gene (5). Dystrophin and its associated proteins are commonly known to be indispensable for the functioning of the intracellular actin cytoskeleton, as are laminins in the extracellular matrix of muscle fibers, which protect myofibers from contraction-induced damage (6). Loss of dystrophin causes the rapid and continuous damage of muscles, which leads to the exhaustion of both skeletal muscles and satellite cells, even though muscular regeneration occurs at a higher frequency in DMD patients than in nonaffected individuals (7). Despite extensive efforts to establish pharmacological agents that halt the clinical course of DMD, the disease still results in high mortality in patients during late adolescence.

Skeletal muscle stem/progenitor cell transplantation is considered to be one of the most promising therapies for the muscular dystrophies. In fact, a recent report has shown that the transplanted satellite cells can engraft as myofibers with normal dystrophin expression in the muscles of *mdx* mice, a mouse model of DMD (8, 9). Most of the clinical trials involving allogeneic transplantation of DMD, however, have not obtained satisfactory results due to immune rejection, rapid death, and limited migration of transplanted myoblasts (10).

Embryonic stem (ES) cells have considerable advantages over somatic stem cells as a cell source of transplantation due to their capacity for unlimited proliferation in an undifferentiated state over a prolonged period, and their ability to differentiate into various lineages of cells in the same way as observed *in vivo* (11). Recently, mouse and human induced pluripotent stem (iPS) cells have been established by introducing 3 or 4 pluripotency-associated genes into somatic cells (12–21). Like ES cells, these reprogrammed somatic cells possess properties of self-renewal and pluripotency, and yield germline adult chimeras. Furthermore, the iPS cell technology enables us to generate individualized stem cells, which is expected to contribute to patient-oriented disease studies, drug screenings, toxicity tests, and transplantation therapies (22, 23).

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