表2 報告されたアルドラーゼ異常症の臨床像と遺伝子変異

	臨床像				変異解析結果		文献
	近親婚	溶血性貧血	筋症状	精神発達遅延	DNA	アミノ酸	
1	あり	あり	なし	あり	ND	ND	1)
2	あり	あり	なし	なし	c.386A>G	p.Asp128Gly	2), 5), 6)
3	なし	あり	あり	軽度あり	c.619G>A	p.Glu206Lys	3)
4	なし	あり	あり	なし	c.931C>T c.1037G>A	p.Arg303X p.Cys338Tyr	4)

り報告された第1例[1]はカナダ在住のユダヤ人男性で，両親はいとこ結婚であった．生下時に鼻・眼・頸部の外表奇形，肝腫大を指摘された．生後6週でHb 7.5g/dL，網赤血球数10％の溶血性貧血を指摘された．その後3カ月間で4回赤血球輸血を受けており，ヘモグロビン(Hb)は4-11g/dL，網赤血球3-16％の慢性溶血を呈した．貧血以外の所見として，低身長や言語発達障害が指摘された．この例の赤血球アルドラーゼ活性は0.46-0.57IU/gHbと正常対照の3.22±0.49に比して約16％に低下を認めた．

第2例は本邦例[2]で14歳の男児である．発端者は満期産で生下時体重は3,140g，新生児黄疸も生理的な範囲であった．生後2カ月時に貧血と肝腫大を指摘された．Hb 5.9g/dL，網赤血球8.4％，間接ビリルビン2.0mg/dLで赤血球像では大小不同および赤芽球を末梢血に認めた．患者赤血球の走査電顕像では摘脾後のピルビン酸キナーゼ(PK)異常症で散見される金平糖(ウニ)状赤血球(echinocytes)を認めた．赤血球アルドラーゼ活性は0.17IU/gHbと基準値2.50-3.36に比して約6％に低下を認めた．

第3例はドイツからの報告例[3]で，4歳半の男児である．この例は筋力低下と易疲労性を主訴に来院した．彼は10分以上の歩行，20段以上の階段を登ることが困難であった．生下時体重は2.75kgで既往歴として光線療法が必要となる重症新生児黄疸があった．原因不明の黄疸と貧血のため，生後1年以内に数回の赤血球輸血を必要とした．初診時筋萎縮，トーヌスの低下および近位筋の筋力低下が著明であった．肝脾腫を認め，体重は−3.4SD，身長は−1.8SDであり，運動および言語の発達遅滞を認めた．Hb 9.7g/dL，網赤血球数6.5％で溶血所見を認め，特記すべき事項として血清クレアチンキナーゼ(CK)が2,620U/Lと著明な高値を呈していた．赤血球アルドラーゼ活性は対照の約10.7％に低下を認めた．患者赤血球ATPは軽度低下，赤血球グルコース消費率は対照の約60％に低下しており，解糖系障害によるATP産生低下が証明されている．患者赤血球アルドラーゼの56℃における熱安定性は著明な低下を認めた．

第4例はイタリアから報告された[4]．シシリア島出身の女児で新生児黄疸およびpyropoikilocytosisを伴う貧血のため赤血球輸血を必要とした．彼女の母親には新生児黄疸の既往があり，赤血球像で楕円赤血球を認めた．父にはスペクトリン異常は同定できず，患児の貧血がhereditary pyropoikilocytosis(HPP)によることは否定的であった．1-3歳まで発端者は反復性の肺炎を発症し，播種性の*Pseudomonas*感染症を認めた．2歳児には痙攣発作があり，Gowers徴候を認め，筋力低下を疑われた．患児は6-8週ごとの赤血球輸血が必要であったため，3歳時に脾臓摘出術を施行され，以後赤血球輸血への依存は消失した．血清CKは13,900U/Lと高く，反面血清アルドラーゼ値は6.2U/Lと相対的に低く，赤血球アルドラーゼ活性は対照の約15％に低下を認めたため，アルドラーゼA異常症と診断された．

3. 遺伝子検査結果と変異酵素の性質

世界で最初に遺伝子解析されたのは日本人症例であり，Kishiらによりコドン128のミスセンス変異，c.386A>Gが同定された[5]．この変異により単一アミノ酸置換Asp128Glyが生じる．

表3　溶血性貧血とミオパチーを合併しうる解糖系酵素異常症

酵素名	OMIM[a]	アイソザイム遺伝子（遺伝子座）	備　考
グルコースリン酸イソメラーゼ	172400	*GPI*（19q13.11）	ペントースリン酸経路の障害を伴い，急性溶血発作を伴う例が多い
ホスホフルクトキナーゼ	232800	*PFKM*（12q13.11）	糖原病VII型（垂井病）赤血球には*PFKL*も発現している
アルドラーゼ	103850	*ALDOA*（16p11.2）	精神発達遅延を伴う例がある一例で好中球機能異常が示唆されている
トリオースリン酸イソメラーゼ	190450	*TPI1*（12p13.31）	精神発達遅延を伴う例がある
ホスホグリセリン酸キナーゼ	311800	*PGK1*（Xq21.1）	X染色体劣性遺伝　精神発達遅延を伴う例がある

[a]On-line Mendelian Inheritance in Man[http://omim.org/].

このAsp128残基は，3つのアルドラーゼアイソザイムで共通のアミノ酸であり，患者赤血球およびリンパ芽球から抽出されたアルドラーゼおよび当該塩基置換を導入後，大腸菌で発現させた後に得られるリコンビナント変異アルドラーゼAが熱不安定性という共通の性質を示した[5,6]ことから，本塩基置換が病因遺伝子変異と考えられた．患者はこの変異に関してホモ接合体，両親はヘテロ接合であった．

次に遺伝子変異が同定されたのはドイツ人小児例で，同定された変異はc.617G>A（Glu206Lys）だった．このGlu206残基はすべてのヒトアルドラーゼに共通であり，アルドラーゼの四量体形成に重要なアミノ酸残基と考えられた[3]．

イタリア人症例にはArg303X，Cys338Tyrの2つの変異が同定され，症例は複合ヘテロ接合体と考えられた．Arg303Xはナンセンス変異であり，Cys338TyrはアルドラーゼのC末端における構造安定性に寄与するCys338に生じたミスセンス変異である[4]．

4. 診断と鑑別診断

新生児期から発症する先天性溶血性貧血で血液型不適合や自己免疫性溶血性貧血などの免疫学的機序による溶血性貧血や赤血球形態異常を伴う赤血球膜異常症が否定されたケースでは，赤血球酵素異常症あるいはHb異常による溶血性貧血を疑う[7]．赤血球酵素異常症では新生児期重症黄疸に対してしばしば光線療法や交換輸血が必要であり，その後引き続いて赤血球輸血を必要とする慢性溶血が生後数カ月以内に認められる．

アイソザイムのない解糖系酵素，赤血球と骨格筋のアイソザイムが同一である解糖系酵素（**表3**）の活性低下では，その後運動発達遅滞，筋力低下やミオグロビン尿を呈することは特記すべき事項である．したがって原因不明の先天性ミオパチー症例では，両親に血縁がないかどうか，ホスホグリセリン酸キナーゼ（PGK）異常症では母方の親戚に同様な症状を認める男性がいるかどうかなどの家族歴を詳細に聴取する必要がある．また明らかなHb低下がなくてもよく代償された溶血があるか否か，網赤血球数，間接ビリルビン，乳酸脱水素酵素（LD），ハプトグロビンなどの溶血関連検査項目を調べる必要がある．

5. 診断と鑑別診断

先天性溶血性貧血については赤血球輸血，脾臓摘出術などの対症療法のみ存在する．赤血球造血を支持するため，葉酸の補充が望ましい．赤血球輸血依存例や無効造血が顕著な例では二次性鉄過剰症を合併するため，血清フェリチン値を1,000 ng/mL未満に保つよう，鉄キレート剤（デフェラシロクス）の内服が必要となる．

アルドラーゼA異常症によるミオパチー発症例は報告例が極めて少ないため，遺伝子検査結果によって個別化医療が提供できるまでには至っていない．今後症例数が蓄積し，アルドラーゼの構造と機能に関する基礎研究が進むことで，変異部位の同定により適切な健康管理情報の提供が可能になることが期待される．

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別冊日本臨牀　新領域別症候群シリーズ　No. 21（2013年1月20日発行）別刷

血液症候群（第2版）

—その他の血液疾患を含めて—

I

II　赤血球の異常

貧血　溶血性貧血　先天性溶血性貧血

赤血球酵素異常による溶血性貧血　Embden-Meyerhof回路

ヘキソキナーゼ異常症

菅野　仁

II 赤血球の異常

貧血　溶血性貧血　先天性溶血性貧血
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ヘキソキナーゼ異常症

Molecular basis of human red blood cell hexokinase deficiency associated with congenital hemolytic anemia

菅野　仁

Key words：先天性溶血性貧血，赤血球酵素異常症，解糖系

1. 概念・定義

ヘキソキナーゼ(EC 2.7.1.1，HK)は解糖系の最初の段階を触媒する酵素であり，ATP存在下でグルコースをグルコース-6-リン酸へリン酸化する．HKにはHK1からHK4の4つのアイソザイムが存在し，その各々が別個の構造遺伝子にコードされている(表1)．先天性溶血性貧血の原因となるのは1型ヘキソキナーゼ遺伝子(*HK1*)の構造変異である．このHK異常症は解糖系酵素異常による先天性溶血性貧血の一型であり，現在までに世界で18家系のみが報告されている極めてまれな疾患である．

2. *HK1*遺伝子にコードされている赤血球特異的HK

ヒト*HK1*遺伝子は10q22.1に遺伝子座があり，全長約131 kbpで27個のエクソンにより構成されている．ほぼユビキタスに発現しているHK1をコードするエクソン1-18以外に，赤血球特異的なエクソンR[1]，精巣(testis)・神経系(neural tissue)特異的エクソンT1～T6，そして最近見つかったAltT2，AltT4の2個で構成されている[2]．

成熟赤血球の主要なアイソザイムはHK1であるが，Murakamiらにより赤血球特異的なR型HK(HKR)が同定された[3]．網赤血球や幼若

表1　ヘキソキナーゼアイソザイム

	1型(HK1)	2型(HK2)	3型(HK3)	4型(HK4，GCK)
遺伝子座	10q22.1	2p12	5q35.2	7p13
構造遺伝子	*HK1*	*HK2*	*HK3*	*GCK*
分子量	100 kDa	100 kDa	100 kDa	50 kDa
Km(glucose)	<1 mM	<1 mM	<1 mM	>1 mM
発現組織	ほとんどの組織(含む赤血球，中枢神経系，精巣)	骨格筋，心筋，脂肪組織	肝臓(間葉系細胞)，腎臓，脾臓，肺，白血球	肝臓(肝細胞)，膵臓β細胞
活性低下による疾患	先天性溶血性貧血，胎児水腫，HMSNR*	知られていない	知られていない	糖尿病(maturity-onset diabetes of the young: MODY)
OMIM	*142600	*601125	*142570	*138079

*HMSNR: Hereditary motor and sensory neuropathy, Russe.

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0047-1852/13/¥60/頁/JCOPY

表2 現在までに報告されたヘキソキナーゼ異常症

症 例	診断時年齢	性 別	Hb(g/dL)	網赤血球(%)	赤血球HK活性 (%of normal mean)
1	0	F	9.4	13	63
2	38	F	(Ht 36%)	7-13	79
3	22	M	ND	1.7-6.3	49
4	2	M	6.5	5	75
5	28	F	8.5-9.6	2	39
6	30	F	11.3	39	53
7	48	M	12-13.5	10-18	163
8	2	M	8.6-9.4	6.7-8.5	53
9	11	F	11.6	5	83
10	9	F	8.7	12	68
11	1	F	7.0-9.2	3.1-8.1	48
12	19	M	13.8	33	180
13	7	M	9.7	4	70
14	19	F	9.8	50	25
15	27	F	5.3	1	89
16	1	M	11.6	3	45
17	胎児(32週)	F	3.7	42	17
18	33	M	8.8	1.8	61

表3 *HK1*遺伝子変異による疾患と原因遺伝子変異

	変異1	変異2	表現型	OMIM
1	ミスセンス変異 c.1667T>G (Leu529Ser)	遺伝子内欠失 (エクソン6) (del 577-672)	溶血性貧血	*142600
2	ミスセンス変異 c.2039C>G(Thr680Ser)		溶血性貧血	
3	遺伝子内欠失(エクソン5-8)		非免疫性胎児水腫	
4	ミスセンス変異 c.278G>A (Arg93Gln)	赤血球特異的 プロモーター変異 (−193A>G)	溶血性貧血	
5	イントロン変異 (エクソンAltT2下流75bp, G>C)		遺伝性運動感覚ニューロパチー	%605285

な赤血球にはHKRが認められるが赤血球の加齢に伴い発現量は少なくなり，HK1が主要なアイソザイムとなる．このアイソザイムのスイッチングの生理的な意義は不明である．*HK1*には赤血球特異的なプロモーターとそれ以外の組織に広く発現のあるプロモーターがあり，エクソンRは赤血球特異的，エクソン1はそれ以外の組織におけるユビキタスなプロモーターにより使い分けられる．エクソンRには赤血球特異的なアミノ酸配列が含まれており，代わりにその他の組織ではエクソン2由来のアミノ酸配列がコードされている．

3. 遺伝子解析結果/変異酵素の性質と貧血の重症度(表2，3，図1)

HK異常症の本邦第1例[4]は胎生29週女児で子宮内胎児発達遅延および著明な胎児貧血を認めた．患児の赤血球HK活性は正常対照の約15

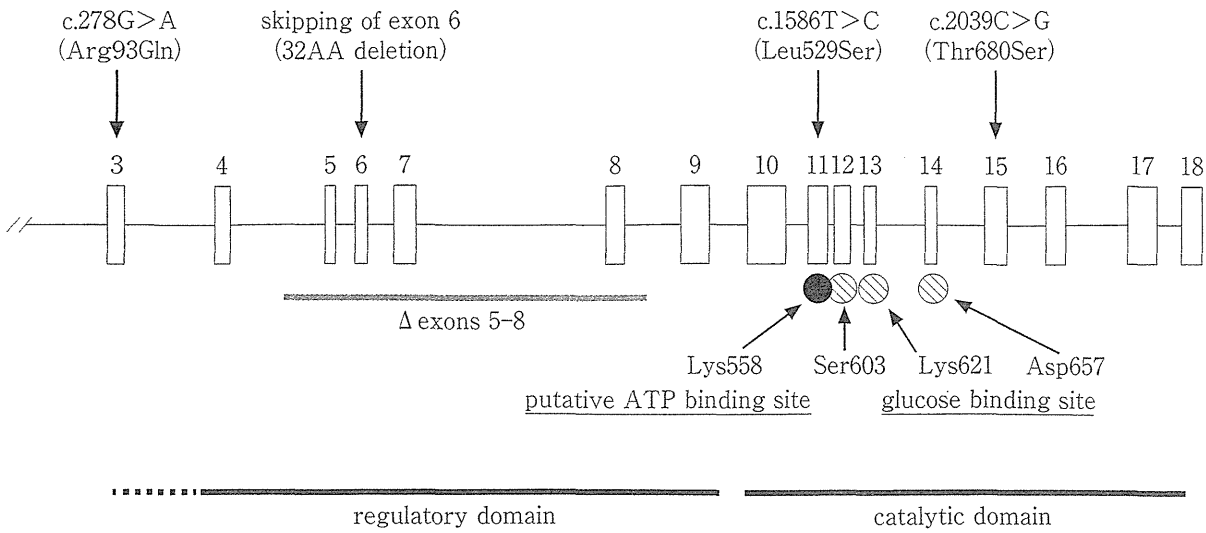

図1　ヘキソキナーゼ異常による先天性溶血性貧血症例で同定された HK1 構造変異

ユビキタスに発現する HK1 をコードする *HK1* 遺伝子はエクソン 1–18 の 18 エクソンにより構成されている．エクソン 1–9 と 10–18 の構造はよく似ており，元は 9 個のエクソンから構成されていた先祖遺伝子の重複により生じたことが示唆される．酵素活性を有する catalytic domain はエクソン 10 以降にコードされており，基質となるグルコースおよび ATP との結合部位が同定されている．HK1 異常症で同定された変異酵素のうち，この領域のミスセンス変異は活性部位の構造変化をきたし，残存酵素活性はほぼ消失する．一方，エクソン 6 の欠失例は in-frame の 32 アミノ酸欠失をきたすが，recombinant HK1 の発現実験により 10％程度の残存活性を認めている．Arg93Gln はプロモーター変異との複合ヘテロ接合体として同定されているが，同定された症例の溶血性貧血は活性中心変異例に比べて軽症である．

%，両親は約 50％に低下しており，HK 異常症と診断した．胎児は脳質周囲白質軟化症を合併し，37 週で子宮内死亡した．患児 *HK1* 遺伝子の各エクソンについて PCR 増幅を試みたところ，エクソン 5–8 のみ PCR 産物が得られず，また網赤血球 mRNA の解析ではエクソン 5–8 領域を欠失する異常 *HK1* cDNA のみが認められた．異常 HK ではフレームシフトが生じ，HK1 と HKR 活性は完全に欠損する．イントロン 4 および 8 の構造解析および患者 *HK1* 遺伝子の欠失部分のクローニングにより，患児は *HK1* 遺伝子の約 9 kbp に達する遺伝子内欠失のホモ接合体であることが明らかになった．

HK Utrecht[5]はオランダのグループから報告された *HK1* のミスセンス変異，c.2039C>G (Thr680Ser)であり，患者はこの変異のホモ接合体であった(症例 14)．Thr680 は HKR の活性中心を構成するアミノ酸残基である．患者赤血球から粗精製された変異酵素は Mg^{2+}–ATP に対する親和性が低下しており，赤血球における HK 活性の低下と矛盾しない結果であった．一方，同じ変異を *HK1* cDNA に導入した組換え型 HK1 を用いた検討では ATP の親和性低下が認められず，同一部位の単一アミノ酸置換が N 末端の構造のみが異なる HKR と HK1 との間で，相反する酵素学的影響を与えている点でユニークと考えられた．

イタリアのグループから解析報告があった HK Melzo[6](症例 15)は，*HK1* 遺伝子変異の複合ヘテロ接合体であった．組換え型 HK1 を用いた検討では，ミスセンス変異 c.1586T>G (Leu529Ser)は HK 活性の完全消失を呈しており，一方 96 bp 欠失をきたすもう一つの変異 del (577–672)は in-frame の 32 アミノ酸欠失をきたす(del 577–672)．この欠失アレル由来の HK 活性は 10％程度残存することが recombinant HK1 の発現実験により明らかとなっている．

また最近オランダのグループから報告された症例(症例 18)は，赤芽球特異的プロモーターの点変異，−193A>G とミスセンス変異，c.278G>A，Arg93Gln，との複合ヘテロ接合体であった[7]．*in vitro* での機能解析実験により，このプ

ロモーター変異は*HK1*遺伝子の転写制御部位における転写因子c-junの結合を阻害することが示された.

前述のように*HK1*遺伝子には精巣・神経系特異的エクソンが同定されていたが, その生理的な意義については不明であった. 遺伝性運動感覚ニューロパチー(hereditary motor and sensory neuropathy: HMSN)の一型である, Russe型HMSN(HMSNR, OMIM%605285)の原因遺伝子が*HK1*であることが明らかになった[1]. HMSNRは*HK1*遺伝子エクソンAltT2の下流75 bpに存在するイントロン部分の点変異によって発症することが同定された. 新しく同定されたAltT2, AltT4は眼球・視神経などに発現しているEST(expressed sequence tag)にも含まれていることから, これらエクソンを含む領域の遺伝子変異が未知の神経～眼疾患の原因となっていることも示唆される.

4. 診断と鑑別診断

新生児期から発症する先天性溶血性貧血で, 血液型不適合や自己免疫性溶血性貧血などの免疫学的機序による溶血性貧血や赤血球形態異常を伴う赤血球膜異常症が否定されたケースでは, 赤血球酵素異常症あるいはヘモグロビン異常による溶血性貧血を疑う[8]. 赤血球酵素異常症では新生児期重症黄疸に対してしばしば光線療法や交換輸血が必要であり, その後引き続いて赤血球輸血を必要とする慢性溶血が生後数カ月以内に認められる.

5. 治療と予後

先天性溶血性貧血については赤血球輸血, 脾臓摘出術などの対症療法のみ存在する. 赤血球造血を支持するため, 葉酸の補充が望ましい. 赤血球輸血依存例では二次性鉄過剰症を合併するため, 血清フェリチン値を1,000 ng/mL未満に保つよう, 鉄キレート剤(デフェラシロクス)の内服が必要となる.

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別冊日本臨牀　新領域別症候群シリーズ　No. 21（2013年1月20日発行）別刷

血液症候群（第2版）

—その他の血液疾患を含めて—

I

II　赤血球の異常

貧血　溶血性貧血　先天性溶血性貧血

赤血球酵素異常による溶血性貧血　Embden-Meyerhof回路

三炭糖リン酸イソメラーゼ異常症

菅野　仁

貧血　溶血性貧血　先天性溶血性貧血
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三炭糖リン酸イソメラーゼ異常症

Molecular basis of human triosephosphate isomerase deficiency associated with congenital hemolytic anemia

菅野　仁

Key words：先天性溶血性貧血，赤血球酵素異常症，解糖系，ミオパチー，神経筋症状

1. 概念・定義

三炭糖リン酸イソメラーゼ(triosephosphate isomerase: TPI，EC 5.3.1.1)は，解糖系においてグリセルアルデヒド-3-リン酸(GA3P)とジヒドロキアセトンリン酸(DHAP)の相互変換を触媒する酵素であり，解糖系酵素の中で最も高い活性を有する．

*TPI*の遺伝子座は12p13.31にあり，構造遺伝子*TPI1*(NG 011948)は約4.2 kbpで，7個のエクソンで構成されている．ヒトTPIは分子量54 kDaのホモ二量体であり，サブユニットは248アミノ酸からなる．

赤血球TPI活性低下による先天性溶血性貧血(OMIM+190450)症例は現在までに世界で約40家系が報告されている極めてまれな常染色体劣性遺伝疾患である．我が国ではまだ1例も報告されていない．

2. TPI異常症の臨床像と病態

TPIはユビキタスな酵素であり，すべての組織に発現している．アイソザイムは報告されておらず，その活性低下は多臓器に障害を生じ，多彩な症状を呈する．すなわち，先天性溶血性貧血，進行性の神経筋症状，そして易感染性が主な症状である[1,2]．

溶血性貧血の最初の症状はピルビン酸キナーゼ異常症などの解糖系酵素異常症と同様な新生児重症黄疸である．生後1カ月を過ぎてからは慢性溶血と感染により誘発される急性溶血発作が繰り返される．慢性溶血性貧血はHb 10 g/dL程度と重症ではない[3]．

TPI異常症の神経筋症状は，運動発達遅延，進行性の筋力低下および痙性麻痺などである．ほかにもジストニア様症状，振戦，視神経萎縮などの報告がある．前記のように貧血が軽症のために神経筋症状を契機に受診するケースがあり，注意が必要である．約半数の症例には知能低下が認められている．頻度は少ないが心筋障害を伴った例も報告されている．易感染性はほとんどの患者で認められるが，必ずしも好中球機能低下が認められない．平均寿命は2歳前後であり，死因としては呼吸不全，心不全，重症感染症などが挙げられ，更に原因不明の突然死に至るケースがある．

解糖系酵素異常の中で最も重症な本疾患の病態は十分に理解されていない．赤血球解糖系中間代謝産物の定量を行うと，本疾患ではTPIの基質であるDHAPの著明な蓄積を認める．蓄積した細胞内DHAPによる毒性が多臓器症状の説明となるかどうかはまだ直接の実証が得られていない．

3. TPI異常症の分子異常と赤血球TPI活性および表現型(表1)

最も高頻度に同定されるのが，315C変異で

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0047-1852/13/¥60/頁/JCOPY

表1　*TPI* 遺伝子変異と見いだされた遺伝子型

	変　異	観察された遺伝子型	文　献
1	−5A>G, −8G>A	−5A>G, −8G>A/wild-type[#]	4)
		−5A>G, −8G>A/c.2T>A	5)
2	−5A>G, −8G>A, −24T>G	−5A>G, −8G>A, −24T>G/wild-type[#]	4)
		−5A>G, −8G>A, −24T>G/c.315G>C	5)
3	−43G>A, −46A>G	−43G>A, −46A>G/wild-type	6)
		−43G>A, −46A>G/c.2T>A	6)
4	c.2T>A(p.Met1Lys)	−5A>G, −8G>A/c.2T>A	5)
5	del 86-87GT(frameshift)	del 86-87GT/c.315G>C	5)
6	c.125G>A(p.Cys42Tyr)	c.125G>A/c.315G>C	7)
7	c.218G>C(p.Gly73Ala)	c.218G>C/wild-type[#]	4)
8	c.315G>C(p.Glu105Asp)	−5A>G, −8G>A, −24T>G/c.315G>C	5)
		del 86-87GT/c.315G>C	5)
		c.125G>A/c.315G>C	7)
		c.315G>C/c.315G>C	8)
		c.315G>C/c.511A>G	7)
		c.315G>C/c.568C>T	9)
9	c.367G>A(p.Gly123Arg)	c.367G>A/wild-type[#]	10)
10	c.436G>T(p.Glu146Stop)	c.436G>T/c.721T>C	5)
11	c.463G>A(p.Val155Met)	c.463G>A/wild-type[#]	4)
12	c.511A>G(p.Ile171Val)	c.315G>C/c.511A>G	7)
13	c.568C>T(p.Asp190Stop)	c.315G>C/c.568C>T	9)
14	c.695G>A(p.Val232Met)	**c.695G>A/c.695G>A**	11)
15	c.721T>C(p.Phe241Leu)	c.436G>T/c.721T>C	12)

太字の2種のみがホモ接合体として同定されている．残りの変異はc.315G>Cとの複合ヘテロ接合体として同定されることが多い．

[#] population studyの中で見つかっていて，溶血性貧血や神経筋症状の原因となりうるかどうかは不明である．

あり，当初解析された19症例のうち17症例に同定されている．Glu105は活性中心付近に位置しており，Aspへのアミノ酸置換はサブユニット間の安定性に寄与するArg99，Lys113に影響を与えるため，変異TPIは熱に極めて不安定となる．

4. 診断と鑑別診断

TPI活性低下による先天性溶血性貧血は，赤血球酵素異常症でも最も重症な疾患であり，思春期までに死亡する例が多い．非典型的なミオパチー，ジスキネジア，知能障害を認める症例には，必ず網赤血球数を含む血算，LD，ビリルビン，ハプトグロビンなどの溶血関連検査を実施して，代償性溶血性貧血の存在を確かめる．

赤血球形態に異常を認めない先天性溶血性貧血患者では赤血球酵素異常症や不安定ヘモグロビン症を考慮に入れ，専門機関への検索を依頼する．診断は赤血球TPI活性測定および赤血球内解糖系中間代謝産物定量による．

5. 治療と予後

必要に応じて呼吸管理などの対症療法を行うが，前項のとおり予後不良である．

酵素補充療法が基礎研究として検討されている[13,14]が，まだ臨床応用はされていない．

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Stem Cell Rev and Rep (2013) 9:422–434
DOI 10.1007/s12015-012-9388-1

Mesodermal and Hematopoietic Differentiation from ES and iPS Cells

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Published online: 9 June 2012

Abstract Embryonic stem (ES) and induced pluripotent stem (iPS) cells can differentiate into any type of tissue when grown in a suitable culture environment and are considered valuable tools for regenerative medicine. In the field of hematology, generation of hematopoietic stem cells (HSCs) and mature hematopoietic cells (HCs) from ES and iPS cells through mesodermal cells, the ancestors of HCs, can facilitate transplantation and transfusion therapy. Several studies report generation of functional HCs from both mouse and human ES and iPS cells. This approach will likely be applied to individual patient-derived iPS cells for regenerative medicine approaches and drug screening in the future. Here, we summarize current studies of HC-generation from ES and iPS cells.

Keywords Mesoderm induction · Hematopoietic cell differentiation · ES cell · iPS cell

Abbreviations

ES	Embryonic stem
iPS	Induced pluripotent stem
HSC	Hematopoietic stem cell
HPC	Hematopoietic progenitor cell
HC	Hematopoietic cell

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Introduction

Hematopoiesis is the process by which mature and functional hematopoietic cells (HCs), such as leukocytes (granulocytes, macrophages, lymphocytes), erythrocytes and platelets, are generated from hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) to maintain homeostasis. Hematopoiesis is controlled intrinsically via transcription factors and small RNAs [1–3] and extrinsically through growth factors and extracellular matrices secreted from niche cells surrounding HCs [4–6]. Faulty regulation of hematopoiesis leads to hematological diseases, such as anemia, leukemia and lymphoma, in which HSC transplantation and/or transfusion of erythrocytes or platelets are dependent on disease status. Regardless of the HSC source (patient cells, donor cells, or cord blood cells), transplantation is a promising therapy for some hematological diseases. However, problems remain in HSC transplantation, such as donor shortages, viral contamination and graft-versus host disease. To overcome these problems, HSC generation from other cells is a possible alternative to expansion of cord blood HSCs. Mature HCs, such as erythrocytes and platelets, are obtained primarily from donors and transfused into patients with hematological diseases and under surgical operation. Likewise, use of HSCs is also associated with problems, such as shortage of donors, viral infection and rejection. Overall, the ability to generate mature HCs from other cells would guarantee a continuous supply of cells and ensure safe and efficient transfusion therapy.

To address these issues, in vitro HC generation from other cells, particularly from embryonic stem (ES) cells [7] and induced pluripotent stem (iPS) cells [8], has been attempted. ES cells are established from the inner cell mass of a blastocyst, maintain their pluripotent and undifferentiated status in vitro and differentiate into any cell type in appropriate culture conditions. Furthermore, ES cells can have pluripotency in vivo and form teratoma in immunodeficient mice, and can be used to generate chimeric mice in vivo. To understand molecular mechanisms underlying developmental processes, ES cells are frequently utilized, since they mimic in vivo development in vitro (Fig. 1). iPS cells, on the other hand are created by ectopic expression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) in somatic cells, such as fibroblasts, hepatocytes, gastric epithelial cells, pancreatic cells, B cells and $CD34^+$ cord blood cells, and exhibit properties of pluripotent ES cells. iPS cells established from a patient's somatic cells could function as useful tools for regenerative medicine and drug screening by manipulating lineage specific differentiation in vitro (Fig. 1).

Here we summarize current studies relevant to generation of HCs and mesodermal cells from ES and iPS cells in both mice and humans (Table 1).

General Induction Methods

Several methods to differentiate mesodermal cells and hematopoietic cells from ES and iPS cells have been reported. They include (i) embryoid body (EB) formation, (ii) co-culture with feeder cells, and (iii) growth in extra cellular matrix-coated dishes. In the first method, undifferentiated ES and iPS cell colonies are separated into small cell pieces enzymatically or by physical dissection, following by EB formation in suspension in a culture dish or hanging drop. EBs are spherical cell aggregates that proliferate and differentiate into all three germ layers (i). Alternatively, ES or iPS cells can be seeded on OP9 stromal cells, which are established from the newborn calvaria of op-/op- mice and support HCs differentiation (ii), or seeded on collagen IV-coated dishes, which promote cell proliferation and form

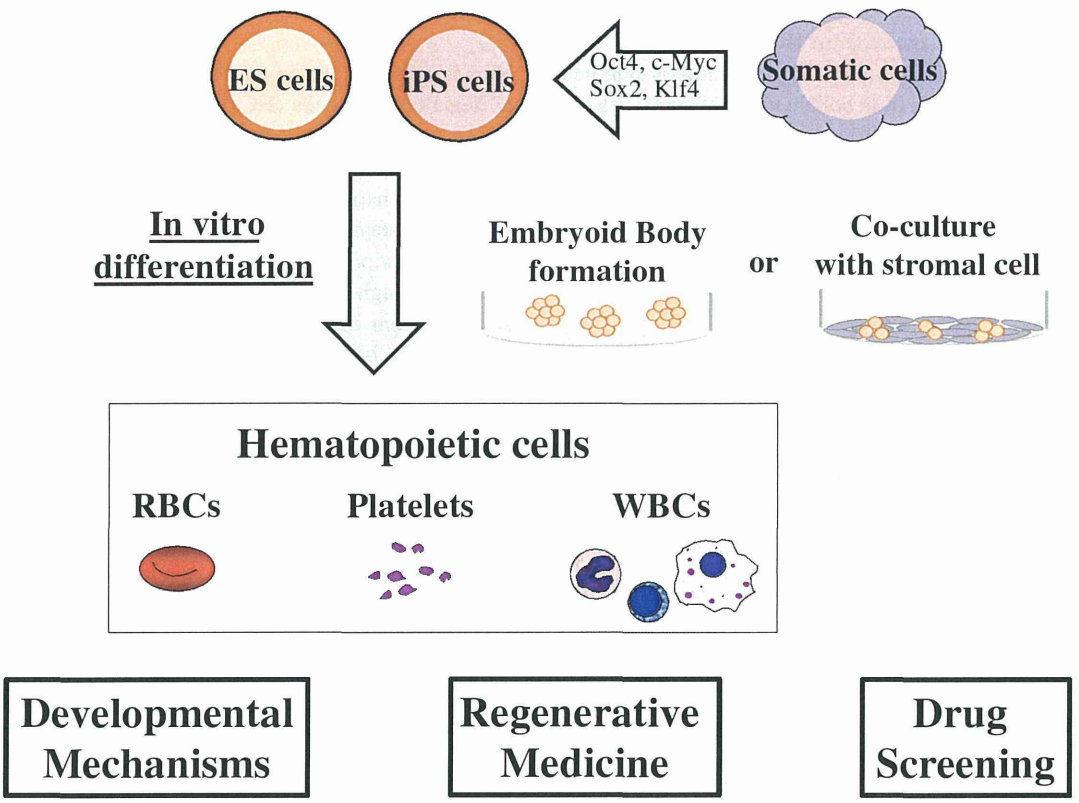


Fig. 1 Generation of hematopoietic cells from pluripotent stem cells in vitro. Embryonic stem (ES) and induced pluripotent stem (iPS) cells have been differentiated into hematopoietic cells (HCs) through mesodermal cells, ancestors of HCs, by two methods: embryoid body (EB) formation and co-culture with stromal cells in vitro. These cells are potentially applicable to regenerative medicine and drug screening by manipulating their lineage specific differentiation in vitro

Table 1 Generation of hematopoietic lineage from pluripotent stem cells

Species	Mouse		Human	
Pluripotent stem cell	ES	iPS	ES	iPS
Mesoderm	12–14	15, 16	17–20	17–20
HPC, HSC	21–23	25, 26	17, 20, 27	17, 20
Erythroid cell	28–32	25	33, 34	17-19, 35–37
Megakaryocyte	38, 39	–	41–43	44
Platelet	40	–	43	44
Macrophage	28, 43–47	48	17, 19, 35, 41	17, 19, 35
Neutrophil	49	–	18, 50, 51	52, 53
Lymphocyte	28, 54, 58, 59, 65	55, 60, 66	56, 61-63, 67	55, 67

an attachment scaffold (iii). In all cases, cell fate, directivity differentiation pattern for mesoderm and HCs differentiation is controlled by cytokines.

Mesodermal Cells

HCs are mesodermal in origin. In mammalian embryogenesis, the three germ layers, ectoderm, endoderm and mesoderm, are formed via spatiotemporal signals. Formation of the primitive streak (PS), the structure that establishes bilateral symmetry, marks the beginning of gastrulation and the emergence of mesodermal precursors. Mesoderm, derived from interaction between the endoderm and ectoderm, forms paraxial, intermediate, and lateral tissues during the mid- to late-streak stage. Among precursors in the axial, paraxial, intermediate and lateral mesoderm, blood vessels and HCs are generated from the lateral mesoderm. Therefore, the appearance of lateral mesoderm is one of indicator to hematopoietic differentiation.

One question is whether ES and iPS cell-derived cells express the surface antigen markers, such as E-cadherin (E-cad), a marker of both ectoderm and endoderm), platelet-derived endothelial growth factor receptor (PDGFRα), and Flk1 (also known as VEGF receptor 2 and a marker lateral mesoderm) [9–11] and transcription factors, such as *Tbx6* and *Brachyury* (a marker pan mesoderm) (Fig. 2).

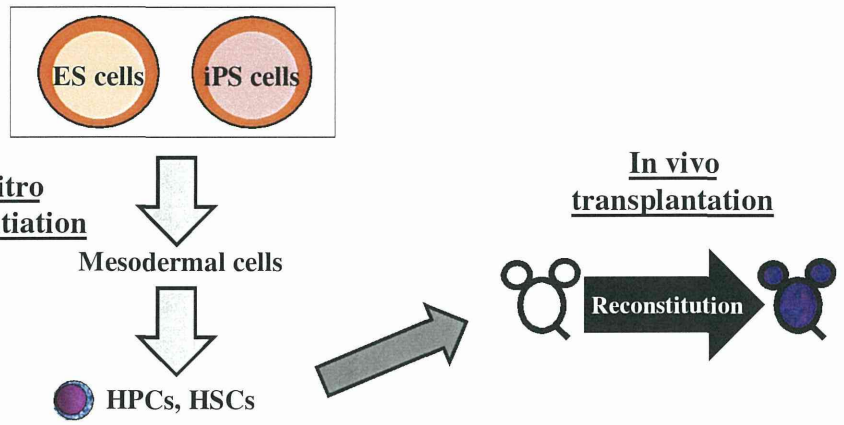
In mouse, a high percentage of $Flk1^+$ mesodermal cells are reportedly obtained from EBs around days 4 to 4.5 after ES cell differentiation [12, 13]. ES cells cultured on collagen-IV-coated dishes differentiate into $Flk1^+PDGFR\alpha^+$ immature mesodermal precursors, which then give rise to $Flk1^+PDGFR\alpha^-$ cells, which are precursor of endothelial and HCs [14]. $Flk1^+$ mesodermal cells have been generated from several kinds of mouse iPS cells, such as mouse embryonic fibroblasts (MEFs), tail tip fibroblasts (TTFs), hepatocytes, and gastric epithelial cells. Among them, MEF-derived iPS cells exhibit the highest proportion of $E\text{-}cad^+Flk1^+$ cells [15]. Mesodermal potential as evaluated by the presence of $E\text{-}cad^+Flk1^+$ cells and expression of *Brachyury, Flk1*, and *Tbx6* mRNA vary among several iPS cell lines derived from identical TTFs. The level of ectopically expressed and remain of c-Myc likely underlies the differences [16].

Some groups have reported mesodermal differentiation from both human ES and iPS cells. $Flk1^+CD34^+$ mesodermal progenitors were generated from KhES1, KhES3 ES cells (KhES1, KhES3) and iPS cells (201B7, 253 G4; derived from dermal fibroblasts) co-cultured with OP9 cells and cytokines [17]. Morishima et al. report that ES (KhES3) and iPS (201B6, 253 G1, 253 G4; derived from dermal fibroblasts) cells generated $Flk1^+$ cells that contained hemangioblasts and $Flk1^{high}CD34^+$ cells with hematopoietic potential [18]. *BRACHYURY* and *WNT3A* mRNA expression was also confirmed in ES-, iPS-, and patient iPS-derived mesodermal cells by others [19, 20].

Hematopoietic Stem (HSCs) and Progenitor (HPCs) Cells

HSCs, which top the hematopoietic hierarchy, have self-renewal capacity and multipotency, and differentiate first into the progenitors of each hematopoietic lineage, which then mature into functional cells, including leukocytes, erythrocytes and platelets. As HSCs are used for transplantation, pluripotent cell-derived HSCs could serve as a source for future clinical applications. Questions remain as to whether HPCs and HSCs derived from pluripotent cells express c-Kit, Sca-1, CD45 in mouse, or CD34 and CD45 in humans in vitro, and whether these precursors have hematopoietic repopulation capacity in vivo (Fig. 2).

In mice, Burt et al. reported a differentiation method that did not require gene modification. EB formation of ES cells treated with SCF, IL-3 and IL-6 with serum for 7–10 days yielded $CD45^+c\text{-}Kit^+$HPCs with long term (for a maximum 20 weeks) repopulation capacity, as measured by chimerism and differentiation into lymphoid and myeloid lineages after transplantation into irradiated mice [21]. However, this approach is not widespread, suggesting that success may depend on serum they used. Therefore, establishment of serum-independent culture condition would be needed to get reproducible result.



Cell types	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Mesodermal cells	FLK1		*Brachyury Tbx6*	*BRACHYURY WNT3A*	Origin of hematopoietic cells and blood vessels
HPCs HSCs	c-Kit Sca-1 CD45	CD34 CD45	*Runx1, Hoxb4, CD41, CD45*	*RUNX1, HOXB4, CD45, CD38*	Hematopoietic reconstitution capacity in transplanted mice (In vivo)

Fig. 2 Schematic diagram of pluripotent cell-derived mesodermal cells, HPCs and HSCs. ES and iPS cells- derived mesodermal cells are characterized by the expression of FLK1 (also known as VEGF receptor 2) and specific transcription factor genes (*Brachyury* and *Tbx6* in mouse; *BRACHYURY* and *WNT3A* in human). Hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs)-derived from pluripotent cells are characterized by the expression of hematopoietic surface markers (c-Kit, Sca-1 and CD45 in mouse; CD34 and CD45 in human) and specific transcription factor genes (*Runx1, Hoxb4, CD41* and *CD45* in mouse; *RUNX1, HOXB4, CD45* and *CD38* in human). In vivo transplantation assay enables us to evaluate the potential of both HPCs and HSCs. Extensive long-term reconstitution with multipotency and self-renewal capacities of all hematopoietic lineages can be observed in HSCs, whereas short and/or relatively long-term reconstituting in HPCs can give rise to certain lineages

On the other hand, to promote the development and expansion of ES cell-derived HSCs, genetically-modified methods have been reported. Kyba et al. reported that forced expression of HoxB4 in immature ES cell-derived HPCs conferred definitive hematopoietic potential in mouse ES cells [22]. Temporal induction of HoxB4 during the course of EB formation increased the number of immature HPCs in vitro. In addition, HoxB4-induced ES-derived HPCs engrafted and produced lymphoid and myeloid cells in both primary (for a maximum 12 weeks) and secondary (for a maximum of 20 weeks) transplanted mice [22]. Combined ectopic expression of HoxB4 and Cdx4, which is also homeobox transcription factor, resulted in more efficient reconstitution than HoxB4 alone [23]. Unlike the case in mouse, a high incidence of leukemia occurred after transducing a *HOXB4*-expressing retroviral vector in large animals, such as dog and monkey [24], suggesting that care should be taken using gene-manipulation methods for HSC transplantation.

The HoxB4-constitutive transduction method is also effective to establish mouse iPS cells from fibroblasts in a sickle cell anemia model. Resulting iPS cell-derived progenitors grown in the presence of OP9 stromal cells reconstituted the hematopoietic system after transplantation into irradiated mice [25]. Lin et al. has reported a hematopoietic differentiation culture method for iPS cells established from MEFs, which lacks a feeder cell layer or gene manipulation. After 7 days of culture with "conditioned medium", EBs prepared from culture of OP9-DL1 cells (modified OP9 cells expressing the delta-like 1 (DL1) Notch ligand) generated c-Kit$^+$Sca1$^+$HPCs that could differentiate along the myeloid lineage [26].It has not yet been examined whether HPCs derived from this method could reconstitute in vivo.

Unlike the case in mouse, ectopic expression of HoxB4 temporarily enhanced generation and proliferation of human HPCs in vitro but did not promote a significant increase in the number of HSCs in vitro and had no effect on repopulating capacity of HSCs in immuno-deficient mice [27]. This

finding suggests that other factors regulate HSC generation from human ES cells.

Importantly, Niwa et al. report that a novel serum-free monolayer culture system, independent of OP9 feeder cells and EB formation enables hematopoietic differentiation from both ES (KhES1) and iPS (201B7, 253 G4) cells. Using the method, the investigators generated $CD34^+CD45^+$ HPCs from human KhES1 between culture days 10 to 25 [17]. Tolar et al. reported hematopoietic differentiation of iPS cells from patients with mucopolysaccharidosis (MPS) type I, which is treated via HSC transplantation. An EB-mediated method generated HPCs expressing CD34 and CD45 and HSCs expressing *CD34* and *CD38* mRNA [20]. It is now necessary to determine whether HPCs derived from gene manipulation-free methods can have repopulation capacity in vivo.

Erythroid Cells

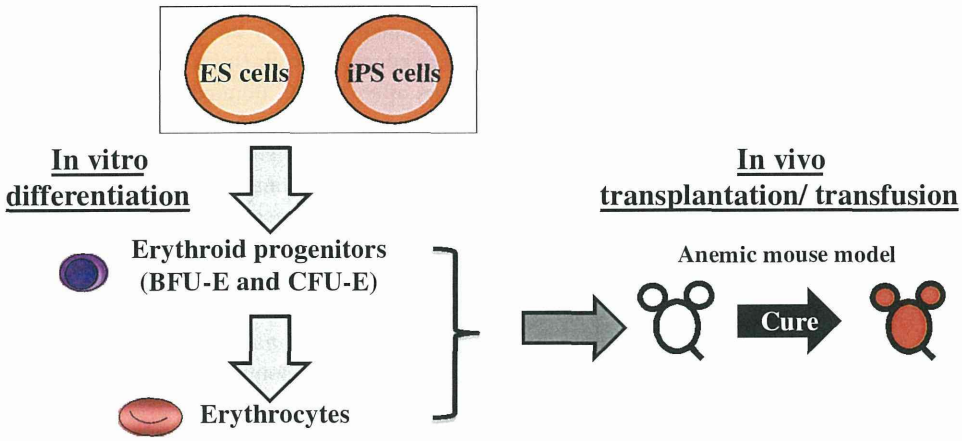
Red blood cells (RBCs), which are differentiated from erythroid progenitors (BFU-E, CFU-E) and erythroblasts, deliver oxygen (O_2) to the body tissues. Pluripotent cell-derived erythroid cells could be utilized as blood products in future clinical applications. It is not yet known whether erythroid cells derived from ES and iPS cells are definitive (EryD), enucleate, generate adult type of hemoglobin or carry oxygen (Fig. 3). In mouse, ES-derived cells co-cultured with OP9 cells contain primitive erythroid cells (EryP) within 8 days and erythroid progenitor cells (EryD) in 10 when cultured in methylcellulose semi-solid culture in the presence of Epo and IL-3. $Ter119^+$ cells were generated in the same condition after 14 days [28–30]. Within 5–6 days, EB culture-derived cells contain erythroid progenitor cells in methylcellulose culture in the presence of Epo and SCF, and those cells show a peak of *βH1 globin*, *βmajor globin* and *Gata1* gene expression within 7–8 days of culture [31].In addition to co-culture and EB formation methods, a three-step differentiation method is highly efficient in inducing erythroid cells from ES cells [32]. After 6 days of EB culture, ES-derived erythroid cultures (ES-EPs), which contain $CD71^+$c-$Kit^+Ter119^+$proerythroblasts and *βmajor globin*-expressing definitive erythroid cells, are induced with Epo, SCF, dexamethasone (Dex), insulin-like growth factor (IGF)-1. Following that, Epo, insulin, glucocorticoid receptor antagonist and transferrin were added to induce hemoglobinized, enucleated RBCs from ES-EP cells that also differentiate into $Ter119^+$ cells in vivo [32]. Hanna's group reported that after 6 days of culture on OP9 stromal cells, mouse autologous iPS cells, which were established from fibroblasts from a sickle cell anemia model, gave rise to c-Kit^+CD41^+ early HPCs in vitro and that HPCs derived by EB formation could rescue anemia in vivo after transplantation [25]. This work suggested that similar strategies are possible in humans.

Human ES cells co-cultured with mouse fetal liver-derived stromal cells (mFLSCs) generated erythroid progenitors after day 10, dominantly express adult type β-globin rather than embryonic type ε-globin, and gradually enucleate and show oxygen saturation around day 16 [33]. Lu et al. report that functional oxygen-carrying erythrocytes can be prepared on a large scale (10^{10}–10^{11} cells per 6-well plate of human ES cells) using four culture steps. They are: Step1; EB formation and hemangioblast precursor induction with BMP4, $VEGF_{165}$, bFGF, SCF, TPO and Flt-3 L (days −3.5 to 0); Step2; hemangioblast expansion with bFGF and tPTD-HoxB4 fusion protein (days 0 to10); Step3; erythroid cell differentiation and expansion with EPO (days 11 to 20); and Step4; enrichment of erythroid cells (day21) [34]. Human fibroblast-derived cell lines, such as hFib2-iPS5, 201B6, 201B7, 253G1 and 253G4, and the bone marrow mesenchymal stem cell-derived MSC-iPS1 cell line, all established from healthy donors, produced erythroid cells at around 30 days of culture via either EB formation or co-culture with OP9 stromal cells [17–19, 35]. On the other hand, two groups reported that human iPS cells from patients with hematological diseases also differentiated into erythroid cells. A combination method using EBs and OP9 cells produced BFU-E progenitor cells from dermal fibroblast-reprogrammed iPS cell lines, which were established from patients with Fanconi anemia (FA) [36]. Human peripheral blood-reprogrammed iPS cells established from patients with polycythemia vera (PV), a myeloproliferative disorder, also differentiate into CFU-E and $CD45^-$/CD235a $(GPA)^+$ mature erythroid cells after 2–3 weeks of EB formation, followed by culture of EB-derived $CD34^+CD45^+$ cells with SCF, IL-3. EPO [37].

Megakaryocytes and Platelets

Platelets, which are derived from fragmentation of precursor megakaryocytes, play a pivotal role in hemostasis by aggregation and adhesion to subendothelial tissue. In terms of regenerative medicine, pluripotent cell-derived platelets could be utilized as blood products in clinical settings. As an experimental tool, pluripotent cell-derived megakaryocytes could be useful to explore signaling pathways utilized in platelet formation, as a genetic approach has limited utility in studying anucleated platelets. One question is whether cells derived from ES and iPS cells would express surface antigen marker, such as CD41 andCD61, have the same signal pathway, exhibit the same morphology and have the same aggregation and adhesion capacity as megakaryocytes and platelets in vivo (Fig. 4).

In mice, Era et al. first reported that megakaryocytes derived from ES cells were observed 8 days after co-culture with OP9 cells and TPO [38].Co-culture of ES cells with OP9



Cell type	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Erythrocytes	Ter119	GPA	*Gata1* *Eklf* *βH1–globin* *βmajor-globin*	*GATA1* *EKLF* *β-GLOBIN*	1) Oxygen-carrying capacity 2) Improvement the anemic status by transplantation and transfusion (In vivo)

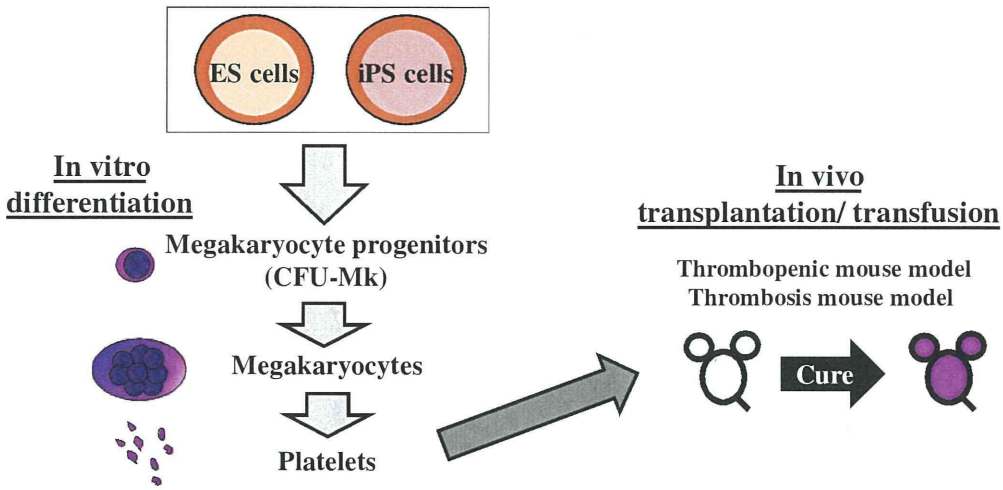
Fig. 3 Schematic diagram of pluripotent cell- derived erythrocytes. ES and iPS cells-derived erythrocytes are characterized by the expression of erythroid surface markers (Ter119 in mouse; Glycophorin A in human) and specific transcription factor genes (*Gata1, Eklf, βH1-globin* and *β−major globin* in mouse; *GATA1, EKLF* and *β-GLOBIN* in human). Hemoglobins in erythrocytes function as an oxygen-carrier and can be evaluated by plotting the oxygen equilibrium curves. To further investigate the function of erythrocytes in vivo, erythrocytes are transplanted/transfused into anemic mouse model. BFU-E; Burst Forming Unit-Erythroid, CFU-E; Colony Forming Unit-Erythroid, GPA; Glycophorin A

stromal cells for 8–12 days in the presence of TPO, IL-6 and IL-11 resulted in development of large, polyploid megakaryocytes, which produce proplatelets, could bound fibrinogen after exposure to platelet agonists, and exhibited integrin-mediated megakaryocyte signaling [39]. Fujimoto et al. confirmed primitive and definitive megakaryopoiesis from mouse ES cells with OP9 stromal cells in the presence of TPO. At the same time, $CD41^+$ platelets were generated in the culture supernatant via two waves of differentiation. ES-derived platelets of the definitive wave are similar in structure to those in peripheral blood and exhibit fibrinogen-binding ability and CD62P (P-selectin) expression after addition of AYPGFK, a platelet agonistpeptide [40].

In humans, 17 days after human H1 ES-derived cells on mouse bone marrow S17 cell lines contained megakaryocyte progenitor cells (CFU-Mk) that expressed CD41 antigen [41]. Gaur et al. reported that human H9 ES-derived cells grown on OP9 cells also generate megakaryocytic cells after 15–17 days of culture with TPO. CD41-, CD42b- and von Willebrand factor (vWF)-expressing megakaryocytes were polyploid (2 N to 32 N) and responsive to integrin $\alpha_{IIb}\beta_3$ activation by agonists such as ADP and PAR1 [42]. Takayama et al. reported a modified method of OP9 co-culture useful to produce not only megakaryocytes but also functional platelets. Differentiated ES cells co-cultured on OP9 or 10 T1/2 cells (a mouse embryonic mesenchymal stem cell line) with VEGF for 14–15 days formed embryonic stem cell-derived sacs (ES-sacs), which consist of multiple cysts that retain properties of endothelial cells and express CD34, VE-cadherin, CD31, CD41a, and CD45. After 9–10 days culture of ES-sacs with TPO, IL-6, IL-11, SCF and heparin, cultures produced functional platelets [43]. The same group succeeded in inducing platelets through "iPS-sacs" from human iPS cell lines reprogrammed from healthy adult human dermal fibroblasts [44].

Macrophages

Macrophages, which are differentiated from monocytes, are amoeboid cells that function in both innate immunity and adaptive immunity, phagocytize bacteria, viruses and dead cells, and stimulate lymphocytes and other immune cells to respond to pathogens. Monocytes and macrophages play a critical role in initiation and progression of atherosclerotic lesions. Therefore, pluripotent cell-derived macrophages could be used in vitro as an experimental tool to clarify the mechanisms of atherosclerotic lesions. It is not known whether macrophages derived from pluripotent cells express



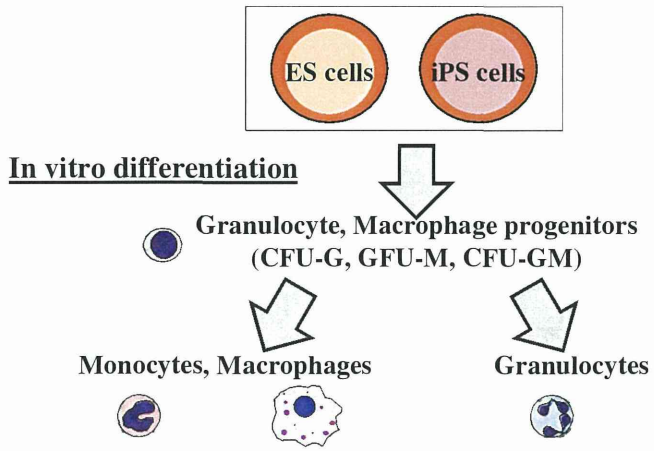
Cell types	Surface markers	Specific genes	Other characteristics
Megakaryocytes	CD41, CD42 CD61	*NF-E2* *FOG-1, Fli-1*	1) DNA ploidy 2) Aggregation and adhesion capacity
Platelets	CD41, CD42 CD61, CD62P	—	1) Aggregation and adhesion capacity 2) Thrombus formation to blood vessel injury (In vivo)

Fig. 4 Schematic diagram of pluripotent cell-derived megakaryocytes and platelets. ES and iPS cells-derived megakaryocytes are characterized by the expression of megakaryocyte surface markers (CD41, CD42 and CD61 in both mouse and human) and specific transcription factor genes (*NF-E2, FOG-1* and *Fli-1* in both mouse and human). DNA ploidy, a representative characteristic of megakaryocytes is examined in CD41 positive cells. Aggregation and adhesion capacity, another characteristic of megakaryocytes, are examined by fibrinogen binding assay and the integrin alpha II beta 3 signaling pathway. ES and iPS cells-derived platelets are characterized by the expression of surface markers (CD41, CD42, CD61 and CD62P in both mice and human). To further investigate the function of platelets in vivo, platelets are transplanted/transfused into thrombopenic mice and injury-induced mice models, in which the improvement of its status and hemostasis can be evaluated, respectively. CFU-Mk; Colony Forming Unit-Megakaryocyte

appropriate cell surface antigen markers, such as CD11b and F4/80, exhibit similar gene expression patterns as normal macrophages, or show phagocytotic activity (Fig. 5).

In mice, 13–15 days after EB formation by ES cells (CCEG2 and D3) in the presence of Epo, IL-1, IL-3, and M-CSF, HCs containing macrophages are generated [45]. Co-culture on OP9 cells with ES (D3) cells for 10 days also induced macrophage progenitors in the presence of IL-3 and Epo [28]. In these reports, macrophages were confirmed by morphologically. Lindmark et al. found that ES-derived macrophages are more similar to those in the peritoneum than mouse macrophage cell lines (J774.A1 and RAW264.7) by DNA microarray analysis [46]. After 10–12 days of culture, ES (J1) cells formed EBs in methylcellulose in the presence of CSF-1 and IL-3. After 3–5 days of liquid culture with CSF-1 and IL-3, differentiated cells expressed F4/80, FcγRII, scavenger receptor A (SR-A), CD36 and CD68, exhibited phagocytosis, and secreted TNF-α and IL-6 in response to an inflammatory stimulus [47], suggesting ES cell-derived macrophages could be an appropriate model to study atherosclerosis-related macrophages. Senju et al. reported that F4/80- and CD11b- expressing macrophages from the mouse iPS cell line 38C-2, which was established from MEFs with Oct3/4, Sox2, Klf4, and c-Myc, were generated via co-culture with OP9 cells for 6 days without cytokines and for the next 6 days with GM-CSF, followed by feeder free culture with M-CSF. iPS-derived macrophages also showed complement C5a-induced chemotaxis, phagocytic capacity and produced nitric oxide after stimulation with LPS and IFN-γ [48].

In humans, 17 days after human H1 ES-derived cells on mouse bone marrow S17 cell lines contained macrophage progenitor cells (CFU-M) that expressed CD15 antigen [35, 41]. Human ES cell lines, such as KhES-1, KhES-3, H1 and HES2, and human fibroblast-derived cell lines, such as hFib2-iPS5, 201B7 and 253G4 and the bone marrow mesenchymal stem cell-derived MSC-iPS1 line, all established from healthy donors, contained CFU-Ms and produced $CD14^+$ monocytes, $CD11b^+$ macrophages (at day14–22) and $CD13^+$ myeloid cells (at day 30) after culture via EB formation or co-culture with OP9 stromal cells in the presence of cytokines [17, 19, 35].



Cell types	Surface markers		Specific genes		Other characteristics
	Mouse	Human	Mouse	Human	
Monocytes Macrophages	CD11b, CD14, F4/80	CD11b, CD13, CD14, CD15, CD18	*MMP-3* *MMP-9*	*PU.1*	1) Phagocytotic capacity 2) Cytokine production
Granulocytes	Gr-1	CD11b, CD13, CD16, CD33	*PU.1*	*PU.1, MPO, CEBPα Lactoferrin Gelatinase*	1) Superoxide production 2) Chemotactic capacity

Fig. 5 Schematic diagram of pluripotent cell-derived monocytes, macrophages and granulocytes. ES and iPS cells-derived monocytes and macrophages are characterized by the expression of surface markers (CD11b, CD14 and F4/80 in mouse; CD11b, CD13, CD14, CD15 and CD18 in human) and specific transcription factor genes (*Metalloproteinase (MMP)-3* and *MMP-9* in mouse; *PU.1* in human). A representative characteristic of macrophages is phagocytotic capacity, in which fluorescence-conjugated *Escheria coli* is administered and its incorporation into macrophages is evaluated by in vitro culture. Also, macrophages can produce cytokine (interleukine-6 and tumor necrosis factor) in response to inflammatory stimulus, such as lipopolysaccharide. ES and iPS cells-derived granulocytes are characterized by the expression of surface markers (Gr-1 in mouse; CD11b, CD13, CD16 and CD33 in human) and specific transcription factor genes (*PU.1* in mouse; *PU.1, Myeloperoxidase (MPO), CEBPα, Lactoferrin* and *Gelatinase* in human). The representative characteristics of granulocytes are superoxide production in response to phorbolmyristate acetate (PMA) stimuli, and chemotactic capacity in response to the chemo-attractant in vitro. CFU-G; Colony Forming Unit-Granulocyte, CFU-M; Colony Forming Unit-Macrophage, CFU-GM; Colony Forming Unit-Granulocyte Macrophage

Granulocytes

Granulocytes, which are derived from common myeloid progenitors and myeloblasts and account for approximately 60 % of leukocytes, are composed of neutrophils, basophils and eosinophils. They contain cytoplasmic granules and play a pivotal role in immunesystem by consuming bacteria and dead cells. Decreases in the number of neutrophils promote infection in several pathological situations, such as leukocyte function deficiencies or myelosuppression caused by chemotherapy. Granulocyte transfusion therapy is considered effective for infections unresponsive to conventional antimicrobial therapies in severely neutropenic cancer patients. Therefore, pluripotent cell-derived neutrophils could be used for such procedures. Thus it is important to determine whether neutrophils derived from pluripotent cells express appropriate markers and exhibit behaviors such as chemotaxis and phagocytosis (Fig. 5).

Leiber et al. reported that mouse CCE ES cell-derived neutrophils are generated after 8 days of EB culture, followed by 7 days of co-culture with OP9 cells and cytokines. The co-culture system is more effective in enhancing the number and culture period of neutrophils produced from ES cells compared to methods that do not employ OP9 stromal cells. Those neutrophils are positive for the surface marker Gr-1, have granules containing lactoferrin and gelatinases, and exhibit chemotactic responses and superoxide production [49].

Several groups have reported neutrophil induction from human ES cells. EB formation of human KhES cells grown in the presence of OP9 cells and cytokines gives rise to CD15-, CD11b- (both neutrophil and monocyte markers) and CD16- (mature neutrophil marker) positive cells at day13 similar to neutrophils found in peripheral blood. They also exhibit the oxidative burst function in killing microorganisms and phagocytotic activity in vitro [50]. Saeki et