Divergent roles of tumor suppressor gene Klf6 in ES cell differentiation

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SUMMARY

Klf6 is a zinc finger transcription factor that has been identified as a tumor suppressor gene inactivated in several human cancers. We previously reported that Klf6^{-/-} mice are embryonic lethal because of impaired hematopoietic differentiation. In that study, we also found Klf6^{-/-} mice have undefinable livers. Combined with this observation and its developmental expression pattern, Klf6 might have a role in differentiation of non-hematopoietic tissues. The aim of this study was to more clearly define the roles of Klf6 in tissue specification using tissue-specific ES cell differentiation systems. We have evaluated three different culture conditions to induce ES cell differentiation, comparing the responses between Klf6^{+/+} and Klf6^{-/-} ES cells. Neuronal differentiation of Klf6^{-/-} ES cells was impaired based on marker gene expression and morphology. Interestingly, Klf6^{-/-} ES cells began to express Hnf3 β mRNA, which identified these cells as endodermal precursors, suggesting that Klf6 ordinarily suppresses endoderm differentiation in this culture condition. Additionally, when Klf6^{-/-} ES cells were grown in StemPro34 serum free medium, they showed enhanced cardiac differentiation to a greater extent than Klf6^{+/+} ES cells, suggesting that Klf6 may have an inhibitory activity in preventing cardiomyogenesis. These findings indicate divergent and tissue-specific roles of Klf6 in ES cell differentiation.

Key words: Klf6, ES cell, differentiation.

INTRODUCTION

Klf6 is a zinc finger transcription factor that has been identified as a tumor suppressor gene inactivated in several human cancers¹⁻³. We previously reported that Klf6^{-/-} mice are embryonic lethal by day 12.5 post fertilization. Their lethality was associated with markedly reduced hematopoiesis and poorly organized yolk sac vascularization. We confirmed this role of Klf6 in differentiation using Klf6^{-/-} embryonic stem (ES) cells, which displayed broad defects in differentiation⁴. However, developmental expression of Klf6 is also noted in several neuronal sites, hindgut, heart, lung, kidney and limb buds⁵. These observations suggested that Klf6 might have broad and divergent roles in tissue differentiation. The aim of this study was to more clearly define the roles of Klf6 in tissue specification,

using ES cell differentiation systems that specifically confer tissue specific differentiation.

MATERIALS AND METHODS

ES cell culture

ES cells were maintained on irradiated mouse embryonic fibroblast (MEF) feeder cells in Dulbecco's modified essential medium, supplemented with 15% fetal calf serum (FCS), penicillin, streptomycin, and 2% leukemia inhibitory factor (LIF) and 1.5×10^4 monothioglycerol (MTG; Sigma-Aldrich, St Louis, MO). Prior to the initiation of differentiation, ES cells were passaged twice on gelatin-coated dishes in the same media to deplete the population of MEF feeder cells.

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Abbreviations: ES cell, embryonic stem cell; EB, embryoid body; FCS, fetal calf serum; IMDM, Iscoves modified Dulbecco medium; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; MTG, monothioglycerol; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.

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Induction of differentiation

We examined three different culture conditions to induce ES cell differentiation. The culture protocols are schematically indicated as Fig. 1. We generated embryoid bodies (EBs) in all culture conditions. For the generation of EBs, ES cells were trypsinized into single-cell suspensions and plated at variable densities (300–4500 cells/ml) into each differentiation media.

Culture condition I is for hematopoietic differentiation. ES cells were initially grown in Iscoves modified Dulbecco medium (IMDM) supplemented with 15% FCS, 5% Protein Free Medium (PFMHII; Gibco/BRL, NY), 2 mM L-glutamine (Gibco/BRL, NY), 6 µl/ml Transferrin (Roche, Switzerland), 0.5 mM ascorbic acid (Sigma-Aldrich, MO), and 4.5×10^{-4} M MTG in 60-mm Petri grade dishes.

In the culture condition II, we induced ES cells to differ-

Fig. 1. Schematic diagram of culture conditions used in this study.

ES cells were maintained with LIF contained medium to inhibit spontaneous differentiation until day 0.

ente into a neuronal lineage. EBs were generated in StemPro34 (Gibco/BRL, NY) supplemented with 2 mM L-glutamine, 0.5 mM ascorbic acid, and 4.5×10^{-4} M MTG. At day 2 of differentiation, EBs were put into medium, which contains 85% of IMDM and 15% of Serum Replacement medium was supplemented with L-glutamine, ascorbic acid and MTG. At day 6, EBs were replated onto a new gelatinized 6 well plates in IMDM supplemented with 15% FCS, L-glutamine, ascorbic acid, and MTG. This allowed EBs to adhere and differentiate on the culture surface. The appearances and rates of colonies with neurite outgrowth were assessed at day 10. Lastly, we used StemPro34 supplemented with 2 mM L-glutamine, 0.5 mM ascorbic acid, and 4.5×10^{-4} M MTG for the culture condition III. We generated EBs in this media, and cultured them for 13 days. At day 13, the percentage of beating EBs was assessed, and EBs were harvested to examine gene expression. All cultures were maintained in a humidified chamber in a 5% CO₂/air mixture at 37°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from undifferentiated or differentiated ES cells using RNeasy kit (Qiagen, CA), and cDNAs were synthesized from 1 µg of total RNA using an RT kit (Promega, WI). Sequences of PCR primers, annealing temperatures, number of cycles, and the sizes of PCR products for each target are indicated in Table 1. Amplified products were visualized by ethidium bromide staining after agarose gel electrophoresis.

RESULTS

In this study, we utilized three different culture condi-

Table 1. PCR primers and condtns.

Gene Name	Forward	Reverse	Annealing	Cycles	Product size
Klf6	TATCTTCAGGATGAGCCCTGCTAC	AGACTTCACCAATGGGATCAGAGG	60	30	276
Rex1	CGTGTAACATACACCATCCG	GAAATCCTCTTCCAGAATGG	60	35	129
E-ras	TTGGGCACAGTGCAAAGATG	GTCATGGTCTTTCACGAAGC	57	35	130
Oct4	AAAGGTGTTACGCCAGACCA	CAGGGTCTCCGATTTCGATA	57	35	151
Fgf5	GTGTCTCAGGGGATTGTAGGAATACG	GTGAAGGAAAGTTCCGGTTGC	65	35	314
Brachyury	CATGTACTCTTCTTGCTGG	GGTCTCGGAAAGCAGTGGC	60	35	313
Gata1	CATTGGCCCCCTGTGAGGCCAGAGA	ACCTGATGGAGCTTGAATAGAGGC	60	35	289
Hnf3β	TGGTCACTGGGACAAGGGAA	GCAACAACAGCAATAGAGAAC	55	35	288
Hnf4α	TTCTGCGAACTCCTTCTGGA	AGGAGCAGCACGTCCTTAAA	55	35	112
Ttr	CTCACCACAGATGAGAAG	GGCTGAGTCTCTCAATTC	55	30	225
Afp	TCGTATTCCAACAGGAGG	AGGCTTTTGCTTCACCAG	55	30	173
Alb	GCTACGGCACAGTGCTTG	CAGGATTGCAGACAGATAGTC	55	35	260
Pax6	GCTTCATCCGAGTCTTCTCCGTTAG	CCATCTTTGCTTGGGAAATCCG	60	40	312
Wnt1	GATTGCGAAGATGAACGCTGTTTC	TCCTCCACGAACCTGTTGACGG	54	40	266
NeuroD	ATGACCAAGCGCGCCTAGA	ACAGGACAGTCACTGTACGCAC	60	35	866
Nkx2.5	CAGTGGAGCTGGACAAAGCC	TAGCGACGGTCTGGAACCA	58	35	216
aMhc	GGAAGAGTGAGCGCCATCAAGG	CTGCTGGAGGTTATTCTCTCG	58	35	302
Mef2C	AGCAAGAATACGATGCCATC	GAAGGGGTGGTGGTACGGTC	58	35	311, 407, 431
β-actin1	TGGCATTGTTACCAACTGGG	TGGATGGCTACGTACATGGC	57	25	192

tions to induce ES cell differentiation, assessing the impact of KLF6 allelic loss. As previously reported we employed a hematopoietic differentiation system⁴⁾, documenting impaired differentiation in KLF6^{-/-} clones (Fig. 2). Instead, there was extended expression of marker genes for the undifferentiated state, including Rex1⁶⁾, E-ras⁷⁾, and Oct4⁸⁾, and further differentiation was clearly impaired in Klf6^{-/-} ES cells. In this culture condition, expression of TTR⁹⁾, and Pax6¹⁰⁾, were also not detected, indicating lack of differentiation into ectoderm and endoderm. Interestingly, the phenotype of Klf6^{+/-} ES cells was at the level intermediate between Klf6^{+/+} and Klf6^{-/-}, suggesting a positive and essential effect of Klf6 in hematopoietic differentiation.

Next, we drove ES cells to differentiate into a neuronal lineage using serum free conditions (Fig. 3). At 10 days of culture differentiation, Klf6^{+/+} ES cells generated EBs with neurite outgrowth. On the other hand, most of Klf6^{-/-} ES cells failed to generate EBs with neurite outgrowth, and Klf6^{+/-} EBs showed an intermediate phenotype between Klf6^{+/+} and Klf6^{-/-} (Fig. 3a–d). In contrast, RT-PCR revealed that markers for ectoderm differentiation, such as Pax6, Wnt1¹¹⁾ and NeuroD¹²⁾, were expressed even in Klf6^{-/-} EBs. Interestingly, expression of Hnf3 β without brachyury was observed in Klf6^{-/-} EBs, but not in Klf6^{+/+} EBs (Fig. 3e), suggesting that Klf6 suppresses endoderm differentiation, but not hematopoietic differentiation in this culture condition.

Finally, in the culture condition III, we cultured ES cells in serum free medium (StemPro34) for 13 days. On day 13, rhythmically beating EBs consistent with a cardiomyocyte phenotype were frequently observed in Klf6^{-/-} EBs. The frequency of beating EBs was about 8 times higher in Klf6^{-/-} EBs than in Klf6^{+/+} EBs (Fig. 4a). RT-PCR showed the

stronger expression of Nkx2.5^{13–15)} and α Mhc^{15, 16)}, both of which indicate cardiac differentiation, while Mef2c, which is a critical regulator of myogenesis, and cardiac development^{15, 17)}, was similarly expressed. We observed not only cardiac markers, but also endoderm and hepatic markers in those EBs. Interestingly, Klf6^{-/-} EBs showed stronger expression of Hnf3 β , Hnf4a, Afp, and Alb than Klf6^{+/+} EBs.

DISCUSSION

In this study, we have observed divergent effects of Klf6 in tissue specific differentiation depending on the tissue was being generated from ES cells. Specifically, whereas Klf6 is required for hematopoietic and neural differentiation, its loss appeared to accelerate cardiomyocyte differentiation, suggesting it ordinarily displays a suppressive effect on this lineage.

In general, Klf6^{-/-} ES cells differentiate slower than Klf6^{+/+} ES cells. Combining the previous observation of ubiquitous expression of Klf6^{5, 18, 19)}, this transcription factor might be required in the normal differentiation processes of many tissues. However the precise time during tissue specific differentiation at which Klf6 is required is like to differ.

Klf6^{-/-} ES cells, cultured in condition I, which induced hematopoietic differentiation, expressed Hnf3 β , a marker of endoderm precursor cells, on the 3rd day of differentiation, when brachyury positive mesoderm precursor cells arise (Fig. 2), then it is downregulated as cells differentiated into hematopoietic lineage (Fig. 2). Kubo et al. showed Brachyury positive cells can express Hnf3 β , and these cells can differentiate into endoderm in a culture condition²⁰⁾. These findings support the existence of mesendoderm,

Fig. 2. Involvement of Klf6 in hematopoietic differentiation.

Differentiation of Klf6^{-/-} ES cells differentiate slower as previously reported. No endoderm and ectoderm marker differentiation are observed, which suggests Klf6 promote hematopoietic differentiation, but it does not suppress endoderm and ectoderm differentiation.

Fig. 3. Klf6 has a critical role in neuronal differentiation.

a) At 10 days of in vitro differentiation, Klf6^{+/+} ES cells could generate EBs with neurite outgrowth. b) Klf6^{+/-} EBs showed impaired neurite outgrowths. c), d) Klf6^{-/-} ES cells failed to generate EBs with neurite outgrowth. e) RT-PCR revealed that markers for ectoderm differentiation, such as Pax6, Wnt1 and NeuroD, were expressed even in Klf6^{-/-} EBs. Expression of Brachyury was not observed in Klf6^{+/+} and Klf6^{+/-} EBs, while expression of Hnf3 β was observed in Klf6^{-/-} EBs, but not in Klf6^{+/+} EBs.

Fig. 4. Negative involvement of Klf6 in myocardial differentiation.

a) Rhythmically beating EBs consistent with a cardiomyocyte phenotype were frequently observed in Klf6^{-/-} EBs cultured in StemPro34 for 13 days. b) Klf6^{-/-} EBs expressed cardiac and hepatic markers stronger than Klf6^{+/+} EBs.

which is a common precursor of mesoderm and endoderm. After the mesendoderm stage, with progression of hematopoietic differentiation, further endoderm differentiation is suppressed, which is indicated by the down regulation of Hnf3 β and the absence of TTR expression. This phenomenon was similarly observed regardless of Klf6 status, while hematopoietic differentiation was clearly impaired in Klf6^{-/-} EBs. This result suggests that in the culture condition I, the contribution of Klf6 is mainly to guide hematopoietic differentiation, and nothing to do with endoderm differentiation.

When Klf6^{-/-} ES cells were cultured conditions that promote neuronal differentiation, neurite outgrowths were clearly reduced, although expression of neuronal marker genes were similar. These findings suggest that Klf6 may primarily involve morphological development of neuronal tissues. Interestingly, Klf6^{-/-} ES cells expressed HNF3 β in this condition, suggesting one of the roles of Klf6 in neuronal differentiation may be suppression of other lineage differentiation including hepatic differentiation. On the other hand, no expression of brachyury in Klf6^{+/+} and Klf6^{-/-} EBs suggests Klf6 does not suppress mesoderm differentiation in the condition II.

Klf6^{-/-} ES cells differentiated into cardiac muscle cells more than Klf6^{+/+} ES cells, which, as noted above, suggesting that Klf6 exerts a suppressive effect on myocardial differentiation in the condition III. The number of beating EBs was significantly increased in Klf6^{-/-} ES cells, and expression of Nkx2.5 and aMhc were stronger than in Klf6^{+/+} EBs. However, further refinements in specific culture conditions are required to definitively address the role of Klf6 in myocardial differentiation.

Collectively, these results indicate that Klf6 is involved in tissue specification through induction and/or suppression of lineage differentiation. While underlying mechanisms are

unclear, an additional element worth considering is the contribution of Klf6 splice forms to their activity during development. Klf6 is the only Kruppel-like factor, in which splicing variants are reported. These alternative transcript products suppress the full length Klf6 function by competitive inhibition^{21, 22}. While splice forms have not yet been uncovered in a development system, this question merits further analysis.

In conclusion, these findings indicate divergent and tissue-specific roles of Klf6 in ES cell differentiation. The promotive and suppressive effects of Klf6 in lineage-specific differentiations are clearly complex and are likely to depend on both the temporal and spatial context of the specific micro-environment.

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