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SHORT COMMUNICATION

Immunoevasion rather than intrinsic oncogenicity may confer MSCs from non-obese diabetic mice the ability to generate neural tumors

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Introduction

Mesenchymal stem cells (MSCs) have generated a great amount of interest in the field of regenerative medicine and for management of inflammation-related disorders [1–5]. Multiple clinical trials utilizing MSCs in the treatment of human diseases (e.g., type 1 diabetes, myocardial infarction, graft versus host disease) have demonstrated their efficacy [6–8], and transplanted MSCs have been shown overall to be well tolerated without adverse effects. Nevertheless, concerns have been raised over the potential risk of MSC-induced tumor development [9–11], while long-term screenings of patients in MSC-based trials are still lacking. We previously observed the development of tumors in murine recipients of syngeneic MSCs obtained from NOD mice, but not with BALB/c-MSCs [12]. The present report aims to elucidate the degree to which malignant transformation is attributable to MSC evasion of recipient immunosurveillance. This question is of paramount importance in order to ensure safe administration of MSCs for therapeutic applications. Furthermore, these data may shed light on the increased incidence of tumors including neural tumors in diabetic patients [13].

Materials and methods

A full description of the experimental procedures can be found in Supplemental Information [14, 15].

Murine MSCs were intravenously injected into recipient mice. PTEN inhibition in NOD mice was achieved by SF1670 treatment. hGH-expressing NOD-MSCs were injected into CTLA4-Ig-treated NOD or control mice, and hGH expression was monitored by ELISA. pAKT was analyzed at steady-state or following in vitro SF1670-

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mediated PTEN inhibition. CD73 expression was evaluated by flow cytometry in NOD- and C57BL/6-MSCs, or in C57BL/6-MSCs treated with LY294002 PI3K inhibitor. The present study was conducted in accordance with Institutional Animal Care and Use Committee approval.

Results

NOD-MSCs induce tumor formation

In a syngeneic MSC transplant, MSCs and recipient share an identical genotype, thus providing an immune-privileged environment as compared to allogeneic transplantation. To test differences in tumor onset, we performed MSC injections in both syngeneic and allogeneic recipients. Normoglycemic NOD mice received NOD-, BALB/c- or C57BL/6-MSCs, and tumor frequency was compared. BALB/c mice that received NOD-MSCs or syngeneic MSCs and C57BL/6 mice that received syngeneic MSCs were used as controls. Only mice that received NOD-MSCs developed tumors, while infusion of BALB/c- or C57BL/6-MSCs did not result in tumor formation, regardless of recipient strain (Table 1). At S100 immunohistochemical analysis, tumors manifested a malignant peripheral nerve sheath tumor (MPNST) histology (Fig. 1a). No differences in tumor onset were observed when NOD-MSCs were injected into syngeneic or allogeneic (BALB/c) recipients, while injection of BALB/c- or C57BL/6-MSCs failed to induce tumors in both syngeneic and allogeneic (NOD) recipients. Notably, when NOD-MSCs were injected into hyper-glycemic NOD recipients, in addition to lungs and liver, which were affected in normoglycemic NOD mice, tumor formation occurred in legs and tail of NOD mice (Table 1).

MSC tumorigenicity is enhanced in immunosuppressed recipients

To test the effect of immunosurveillance impairment on MSC-induced tumor development, we injected NOD-MSCs into immunodeficient NOD/SCID mice. Tumor growth was then compared with tumors observed in immunocompetent control recipients. Tumors that developed in NOD/SCID mice showed a markedly higher severity compared to those that developed in control NOD-MSCs (Fig. 1b; Table 1). As further confirmation of the role of immunosurveillance in controlling MSC tumorigenicity, we administered CTLA4-Ig fusion protein to NOD mice after injection of hGH-secreting C57BL/6-MSCs, with hGH secretion utilized as a marker of MSC proliferation and tumor growth. Strikingly, 5 out of 5 mice developed tumors in lungs and liver, while no carcinoma was detected in untreated recipients (Table 1). While hGH production peaked after 6 days from the initial treatment and then gradually decreased to baseline level in the control group, indicating rejection of allogeneic hGH-C57BL/6-MSCs by NOD recipients, a rapid increase in hGH secretion was detected in CTLA4-Ig-treated NOD mice at day 18 (Fig. 1c).

Table 1 Strain and condition dependency of MSC-generated tumors

Donor MSCs source strain	Recipient strain[a]	Injection type	Tumor formation	Tumor severity[b]	Tumor location
BALB/c	BALB/c	Syngeneic	0 out of 5	–	None
BALB/c	NOD	Allogeneic	0 out of 5	–	None
C57BL/6	C57BL/6	Syngeneic	0 out of 5	–	None
C57BL/6	NOD	Allogeneic	0 out of 5	–	None
NOD	BALB/c	Allogeneic	5 out of 5	**	Lungs, liver
NOD	Norm NOD	Syngeneic	5 out of 5	**	Lungs, liver
NOD	Hyper NOD	Syngeneic	5 out of 5	*****	Lungs, liver, legs, tail
C57BL/6	NOD	Allogeneic	0 out of 5	–	None
C57BL/6	CTLA4-Ig NOD	Allogeneic	5 out of 5	**	Lungs, liver
NOD	NOD	Syngeneic	4 out of 4	**	Lungs, liver
PTENi NOD[c]	NOD	Syngeneic	4 out of 4	***	Lungs, liver
NOD	NOD/SCID	Syngeneic	4 out of 4	*****	Lungs, liver

[a] Norm, normoglycemic; Hyper, hyper-glycemic; CTLA4-Ig NOD, NOD mice receiving CTLA4-Ig treatment

[b] Tumor severity score was given according to overall tumor burden, including size and tissue involvement; * minimal; ** low; *** moderate; **** high; ***** severe

[c] NOD-MSCs donor mice treated with SF1670 PTEN inhibitor

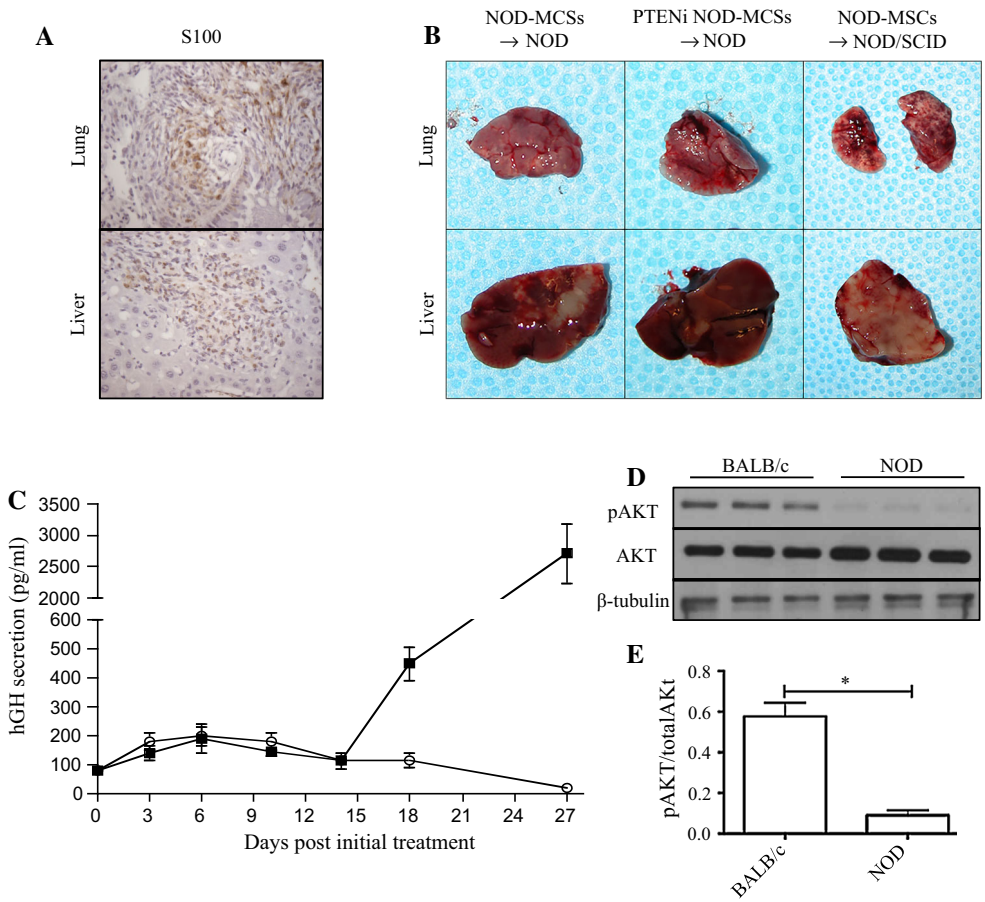

Fig. 1 Oncogenicity features and AKT status of NOD- versus other MSCs. **a** S100 immunohistochemical analysis of NOD-MSC-induced tumors in NOD mice revealed malignant peripheral nerve sheath tumor histology. **b** Effect on tumor growth of AKT hyper-activation and immunodeficiency. NOD-MSC infusion into immunodeficient mice (NOD/SCID, *right*) generated tumors in lung (*upper row*) and liver (*lower row*) of higher severity than those detected in control NOD mice (*left*), even when MSCs with hyper-activated AKT through PTEN inhibition were used (*center*). **c** Human growth hormone (hGH) blood levels in CTLA4-Ig fusion protein-treated (*black squares*) or untreated (*white circles*) NOD mice following infusion of hGH-expressing C57BL/6-MSCs (mean ± SEM, $n = 3$). **d**, **e** Western blot analysis of steady-state pAKT expressed as pAKT/total AKT ratios in NOD- versus BALB/c-MSCs of three independent samples for each strain. *Left bar* BALB/c-MSCs; *right bar* NOD-MSCs. (mean ± SEM, $n = 3$, $^*p = 0.02$) (**e**). hGH, human growth hormone; PTENi NOD, NOD mice treated with PTEN inhibitor SF1670; SEM, standard error of the mean

The oncogenic PTEN/PI3K/AKT axis is not activated in NOD-MSCs

In order to explore the issue of intrinsic oncogenicity of NOD-MSCs, we examined whether the oncogenic PTEN/PI3K/AKT axis is dysregulated in NOD-MSCs. AKT phosphorylation status was assessed by Western blot analysis of NOD- and BALB/c-MSCs, both at steady-state and under in vitro SF1670-mediated PTEN inhibition. We observed a considerable steady-state expression of activated pAKT in BALB/c-MSCs, while it was barely detectable in NOD-MSCs (Fig. 1d, e). pAKT peaked 30 min after SF1670 administration in both NOD- and BALB/c-MSCs (Fig. 2a, b). Importantly, even after PTEN inhibition, the amount of pAKT in NOD-MSCs remained remarkably lower than basal levels of pAKT in BALB/c-MSCs. We then analyzed *Pten* mRNA expression along with its transcriptional inducers (*Atf2*, *Pparγ*) and repressors (*c-Jun*, *Nf-κβ* and *Tgf-β*) in both NOD- and BALB/c-MSCs. *Pten* expression was lower in NOD- as compared to BALB/c-MSCs (Fig. 2c). Intriguingly, *Pparγ*—a *Pten* transcriptional inducer—was markedly higher in NOD than in BALB/c-MSCs (Fig. 2d), while the opposite was observed for the *Pten* repressor *Tgf-β* (Fig. 2e). We then injected NOD mice with NOD-MSCs in which the PTEN/PI3K/AKT oncogenic pathway had been hyper-activated through inactivation of AKT suppressor PTEN function by treating NOD mice with the PTEN inhibitor SF1670 prior to isolation of MSCs. MSCs from AKT hyper-activated donor mice generated tumors of higher severity with respect to control NOD-MSCs (Fig. 1b; Table 1).

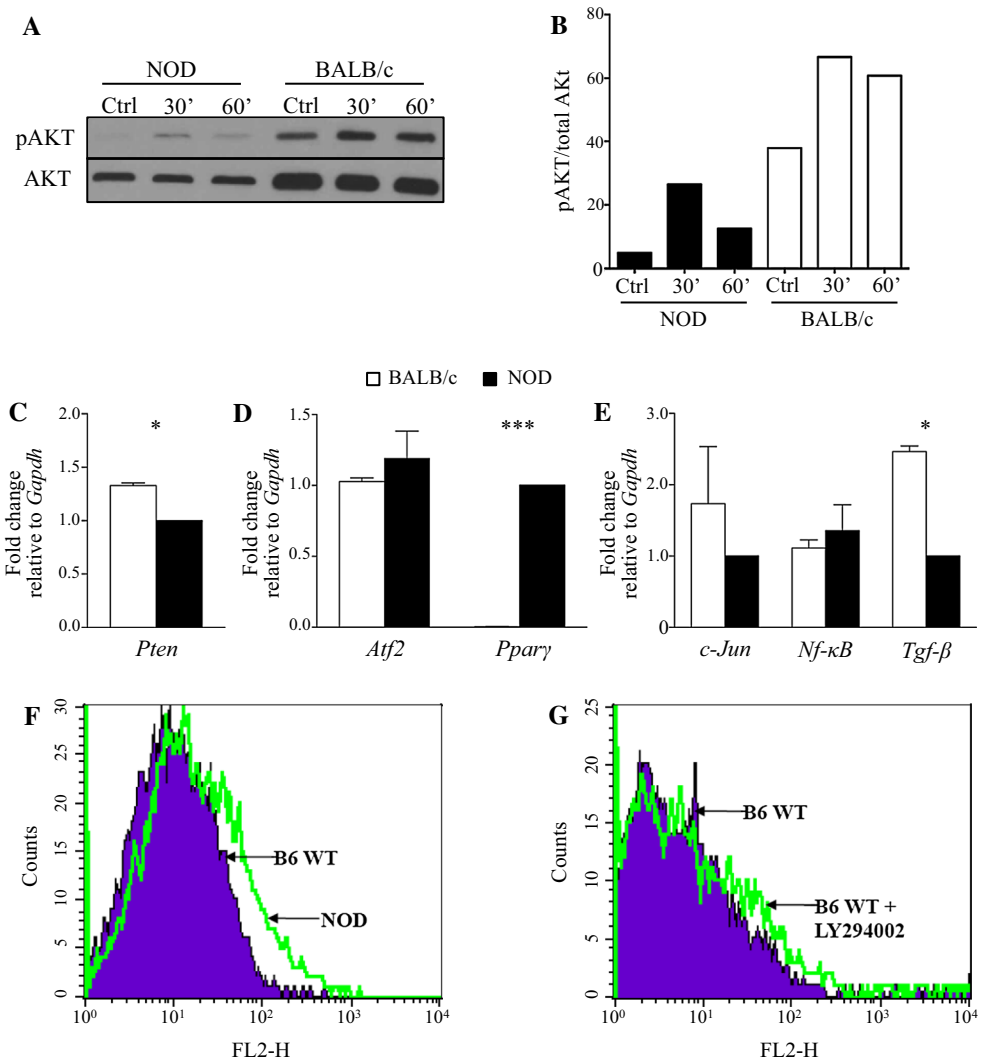

Fig. 2 *Pten* transcriptional status and CD73 expression in NOD- versus MSCs of other origin. **a** Western blot analysis of pAKT expressed as pAKT/total AKT ratios in NOD- versus BALB/c-MSCs following PTEN inhibition with SF1670 at 0, 30 and 60 min after treatment (2 μM). **b** *Solid black bars* NOD-MSCs; *white bars* BALB/c-MSCs. **c** Real-time PCR analysis of *Pten*, **d** *Pten* transcriptional repressors *Atf2* and *Pparγ* **e** and *Pten* transcriptional inducers *c-Jun*, *Nf-κβ* and *Tgf-β* in BALB/c-MSCs (*white bars*) versus NOD-MSCs (*black bars*); data are normalized against *Gapdh* mRNA (mean ± -SEM, $n = 2$, $^{*}p < 0.05$; $^{***}p < 0.001$). **f**, **g** Flow cytometric analysis of CD73 in NOD- (*light green line*) versus C57BL/6- (*violet area)* MSCs (**f**) and in LY294002 PI3K inhibitor-treated (*light green line*) versus untreated (*violet area*) C57BL/6-MSCs (**g**); figures are representative of three independent experiments. Ctrl, non-treated control; SEM, standard error of the mean (color figure online)

NOD-MSCs highly express the CD73 immunosuppressor protein

Once we had established that immunoevasion is a characteristic of NOD-MSCs, we examined whether any immunoregulatory molecules were differentially regulated in NOD-MSCs. We compared CD73 expression using flow cytometry in NOD-MSCs and C57BL/6-MSCs. Our results showed that CD73 is more highly expressed in NOD- as compared to C57BL/6-MSCs (Fig. 2f). We then performed the same analysis in C57BL/6-MSCs in which PI3K was inhibited by LY294002 treatment, and compared CD73 expression in treated and vehicle-treated C57BL/6-MSCs. In PI3K-inhibited C57BL/6-MSCs, CD73 expression was slightly but consistently increased as compared to control C57BL/6-MSCs (Fig. 2g), but was not increased as compared to NOD-MSCs (Fig. 2f). Thus, CD73 immunoregulatory protein expression was higher in NOD-MSCs compared to both untreated and PI3K-inhibited C57BL/6-MSCs.

Discussion

Despite the enormous therapeutic potential of MSCs, concerns exist regarding the risk of long-term tumor development following their infusion, as has been observed

in mice [12]. Herein, we confirmed our previous findings that MSCs from non-obese diabetic mice induce tumor onset. Moreover, we showed that the observed NOD-MSC tumorigenicity is not dependent on recipient mouse strain and that MSCs from non-diabetic prone mice, which are not endowed with such malignant potential, do acquire tumorigenicity in immunosuppressed mice. Histology of tumors detected in NOD mice after syngeneic injection resembled that of MPNST [11]. It has been previously reported that AKT is hyper-activated in these tumors [16] and that the PTEN/PI3K/AKT axis plays a relevant role in diabetic settings [17–19]. Interestingly, NOD-MSCs expressed lower pAKT levels than non-tumorigenic BALB/c-MSCs (Fig. 1d, e), even following AKT repressor PTEN inhibition (Fig. 2a, b). These results suggest that the PTEN/PI3K/AKT axis does not account for NOD-MSC oncogenicity.

According to our alternative hypothesis, NOD-MSCs employ immunoescape mechanisms by circumventing recipient immunosurveillance. Indeed, we found that CD73 is expressed to a higher degree in NOD- compared to C57BL/6-MSCs (Fig. 2f). MSCs express CD73, which dephosphorylates extracellular AMP to adenosine, hence establishing an immunotolerant microenvironment surrounding MSCs [20]. Thus, NOD-MSCs may suppress recipient immune responses by CD73 hyper-expression. Furthermore, the injection of C57BL/6-MSCs into CTLA4-Ig-mediated immunosuppressed NOD mice completely recapitulated the oncogenicity observed with NOD-MSCs (Table 1). Altogether, our data are consistent with the view that NOD-MSC tumorigenicity is favored by immunoescape mechanisms, rather than by their cell autonomous malignant potential.

Other unexplored features of NOD-MSCs play a relevant role in tumor development. For the sake of example, the RAS–MEK–ERK mitogen-activated protein kinase (MAPK) pathway may be also involved in NOD-MSC oncogenicity and is worth investigating in future studies. However, our results clearly indicate that the desirable immunomodulation properties of MSCs also may increase the risk of tumor development in immunocompromised subjects. Finally, tumor incidence has been found to be associated with diabetes and diabetes treatments [13]. Thus, our findings may explain the increased risk of tumor formation in diabetic patients. Further studies on long-term surveillance of patients treated by MSC administration are warranted given the MSC capacity to escape immune surveillance.

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Author's contribution C.L. and R.F.M. designed the study, performed experiments, analyzed data and wrote the paper; M.B.N., S.D., M.B., M.M., V.U., B.E.E., F.D.A., A.O.S.-R. and S.P. performed experiments and analyzed data; G.V.Z. coordinated research; P.F. and R.A. designed the study, provided financial support, wrote and edited the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical disclosure Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, as well as all applicable institutional guidelines for the care and use of experimental animals.

Informed consent This study does not involve human subjects. No informed consent needs to be obtained.

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