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Immune cell recruitment in teratomas is impaired by increased Wnt secretion

Iris Augustin [a,*], Dyah L. Dewi [a], Jennifer Hundshammer [a], Eugen Rempel [a], Fabian Brunk [b], Michael Boutros [a]

[a] German Cancer Research Center (DKFZ), Div. of Signaling and Functional Genomics, and Heidelberg University, Department of Cell and Molecular Biology, Medical Faculty Mannheim, Heidelberg University, Heidelberg 69120, Germany
[b] German Cancer Research Center (DKFZ), Div. of Developmental Immunology, Heidelberg 69120, Germany

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ABSTRACT

Wnt signaling plays a central role in tumor initiation and tumor progression. Mutations in Wnt pathway components, such as the tumor suppressor APC, lead to malignant transformation. While previous studies focused on Wnt-related changes in cancer cells, the impact of aberrant Wnt signaling on the tumor microenvironment is only beginning to emerge. In order to investigate the role of increased Wnt secretion on tumor growth and the microenvironment, we generated a novel germ cell tumor model by overexpressing the Wnt secretion factor Evi/Wls in mouse embryonic stem cells. Evi-overexpressing teratoma were characterized by enhanced tumor growth in supporting a tumor-promoting role of Wnt secretion. Interestingly, enhanced Evi expression correlated with impaired immune cell recruitment. Specifically, T- and B-cell infiltration was reduced in Evi-overexpressing teratomas, which was independent of teratoma size and differentiation. Our study suggests that Wnt secretion impairs immunosurveillance. Since immune cell infiltration has been shown to have prognostic value, the levels of secreted Wnt activity might impact the efficiency of cancer immunotherapy.

1. Introduction

Cells of the tumor microenvironment exert context-dependent pro- and anti-tumorigenic functions (de Visser et al., 2006; Hanahan and Weinberg, 2011). Tumor-infiltrating leukocytes execute critical functions within the complex interactions of tumor cells with their microenvironment. Immunosurveillance mechanisms within the tumor stroma enable the immune system to recognize transformed cells in order to initiate mechanisms of their inhibition or elimination (Dunn et al., 2002; Dunn et al., 2004; Schreiber et al., 2011). During immunosurveillance, cells of the innate and the adaptive immune system cooperate to prevent the growth of neoplastic tissues (Mittal et al., 2014). The cascade starts with an inflammatory signal produced by the tumor cells, which leads to the recruitment of natural killer (NK) cells, macrophages and dendritic cells (DC), followed by T-cell infiltration and activation of their cytotoxic function.

Recent meta-analyses of solid tumors have shown that the $CD3^+$ T cells infiltration correlates with good prognosis (Gooden et al., 2011; Hwang et al., 2012; Mittal et al., 2014; Nakano et al., 2001; Sato et al., 2005; Sharma et al., 2007). However, it is an open question why some tumors are highly infiltrated by T cells and others are not. Recent reports suggest that Wnt/β-catenin signaling in melanoma impairs the antitumor immune response and thereby supports cancer cell-induced immunosuppression (Spranger et al., 2015; Yaguchi et al., 2012). Thus, the better understanding of the involved signaling mechanisms may provide insights into the immune escape mechanisms of tumor cells.

The highly conserved transmembrane molecule Evi/Wls/Gpr177 is an essential component of the Wnt secretion machinery (Banziger et al., 2006; Bartscherer et al., 2006). It is required for the exocytosis of Wnt proteins acting as a Wnt cargo receptor by shuttling between the Golgi, the plasma membrane and endosomal compartments (Bartscherer and Boutros, 2008). Evi is a single-gene family in vertebrates (Jin et al., 2010; Port and Basler, 2010). Thus, it has been assumed that Evi is involved in the secretion of all Wnt proteins. Correspondingly, Evi depletion globally affects Wnt signaling with consequences for canonical as well as non-canonical Wnt signaling. This, in turn, can be exploited as a unique experimental tool to modulate Wnt signaling through the regulation of Wnt ligand secretion. Genetic inactivation of Evi in mice results in embryonic lethality due to disruption of axial patterning with missing mesoderm and primitive streak formation (Augustin et al., 2013; Fu et al., 2009).

Embryonic stem cells (ESCs) are widely used to study early differentiation processes (Posfai et al., 2014). They also hold promise for therapeutic approaches in the field of regenerative medicine (Dressel, 2011; Dressel et al., 2008; Song et al., 2015; Tang and Drukker, 2011). Yet, immune rejection resulting from mismatch histocompatibility is a potential limitation of stem cell-based therapies, restricting the rate of successful transplantation (Zhao et al., 2011; Jin et al., 2015). Moreover,

* Corresponding author at: University of Applied Sciences, Faculty of Biotechnology and Bioinformatics, Am Hofgarten 10, Freising 85354, Germany.
E-mail addresses: i.augustin@dkfz.de, iris.augustin@hswt.de (I. Augustin).

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ESC transplantation may hold the risk of teratoma formation because undifferentiated ESCs give rise to teratomas, which resemble benign tumors that consist of tissues derived from the three embryonic germ layers (Przyborski, 2005; Lee et al., 2013). Accordingly, teratoma formation assays need to be performed to confirm ESC pluripotency in a complex *in vivo* environment. Teratoma assays are also pursued as a model to investigate the crosstalk between ESCs and stromal cells (Przyborski, 2005; Dressel, 2011). We previously generated Evi-overexpressing mouse ESCs (Evi-GOF) *via* knockin into the ROSA26 locus to study Wnt secretion in ESCs (Augustin et al., 2012). Evi-GOF ESCs showed enhanced Wnt activity, which had no effect on ESC pluripotency and viability. Teratoma experiments with Evi-GOF ESCs confirmed pluripotency characterized by meso-, *endo*- and ectodermal lineage differentiation although Evi-GOF ESCs revealed a preference for cardiomyocyte differentiation. Based on these findings, the present study was aimed at investigating Evi during teratoma formation in order to clarify the role of Wnt secretion in teratoma–stroma interactions in a definite genetic setting.

2. Results

2.1. Evi overexpression leads to larger teratomas in syngeneic mice and promotes cardiogenic differentiation

ESCs express a repertoire of Wnt ligands, which activate canonical and non-canonical Wnt signaling cascades. As a cargo-receptor, Evi acts as a gatekeeper to release or block Wnt secretion. Increased levels of the cargo-receptor have been detected in different tumor entities (Augustin et al., 2012; Voloshanenko et al., 2013; Lu et al., 2015). Accordingly, Evi overexpression (Evi-GOF) leads to a global increase of secreted Wnt proteins and represent a model of enhanced Wnt signaling. Evi-GOF ESCs ectopically express Evi-YFP, which is localized to the secretory pathway (Fig. 1A). FACS-based analysis of ESCs confirmed the surface expression of Evi-YFP, indicating proper cellular trafficking of Evi-YFP (Fig. 1B). Western blots of ESC supernatants showed increased Wnt5a/5b secretion of Evi-GOF ESCs, which was abolished in the presence of LGK947 (Fig. 1C). Previous results showed an increased Wnt-reporter activity of Evi-GOF ESCs (Augustin et al., 2012). Accordingly, LRP6, the co-receptor of Frizzled, revealed higher phosphorylation in Evi-GOF ESCs compared to control cells, which was prevented after LGK947 treatment (Fig. 1D). Likewise, expression of the Wnt target gene *Axin2* was increased (Fig. 1E).

Wnt signaling has been shown to support ESC maintenance. Therefore, we investigated the expression of the pluripotency markers *Oct4*, *Nanog*, *Sox2*, *Rex1* as well as Epi-stem cell marker *Claudin1* and early differentiation markers *CD133* and *Fgf5* (Fig. 1F, G). Enhanced Wnt secretion resulted in no significant changes in their gene expression. Similarly, protein expression of Nanog, SSEA1 and Oct4 was unchanged based on FACS analysis indicating no major alterations in the pluripotency network of Evi-GOF ESCs. Moreover, enhanced Wnt secretion had no effect on the viability and growth of cultured Evi-GOF ESCs (Fig. 1H). Similar growth curves were observed in the presence of low serum concentrations (5%) or in the absence of LIF (data not shown).

Subcutaneous injection of Evi GOF and control ESCs in syngenic recipients (129P2/OlaHsd) were performed to generate teratomas and analyze the differentiation potential if ESC *in vivo*. Monitoring of tumor growth over time revealed larger tumors in Evi GOF ESCs (Fig. 2A). Evi-YFP expression was detected in Evi-GOF teratoma sections (Fig. 2B). Labeled cells showed prominent perinuclear and surface staining and were clearly distinguishable from unlabeled host-derived stromal cells (Fig. 2B). Histological analysis of teratomas was performed to assess the differentiation potential of the injected ESCs. H&E stained sections showed characteristic structure of endo-, meso- and ectodermal differentiation (Fig. 2C). Proliferation of tumor cells was not altered as evidenced by EdU-incorporation experiments (Fig. 2D). Active caspase-3 labeling showed reduced apoptosis rate in Evi-GOF teratomas. However, it was not significant. (Fig. 2E).

In order to understand the differences between *in vitro* and *in vivo* growth, we performed expression profiling of teratomas from Evi-GOF and control ESCs. RNA from teratomas was isolated and analyzed by array-based expression profiles to study cellular and molecular mechanisms underlying Evi-GOF teratoma development. A set of 18 genes was significantly upregulated and 123 genes were downregulated in Evi-GOF teratomas (absolute value of $\log_2$ fold change >1; adj. p-value < 0.05) (Fig. 3A). Tissue-related expression analysis of the upregulated genes based on TiGER profiles (http://bioinfo.wilmer.jhu.edu/tiger/) showed that 61% of the genes had a muscle and heart association, whereas 39% of the genes were ubiquitously expressed or had other tissue preferences, supporting the concept that Evi-GOF ESCs reinforced mesodermal differentiation to result in enhanced muscle development (Fig. 3B). Similar results were reported after Wnt1 overexpression (Weisel et al., 2010) or sustained treatment of ESCs with Wnt3a or GSK-inhibitor (Bakre et al., 2007). The list of upregulated genes related to muscle and heart development is summarized in Fig. 3C.

2.2. Reduced expression of immune-modulatory genes in Evi-overexpressing teratomas

Among the downregulated genes, the highest enrichment was observed in genes categorized as immunity and defense (43% of the downregulated genes) as well as signal transduction (30% of the downregulated genes) (Fig. 3D). Similarly, genes involved in T- and B-cell-mediated immunity, as well as innate immunity were differentially expressed suggesting an altered immune response (Fig. 3D). Among others, the genes coding for three CD3 chains (*CD3γ*, *CD3δ*, *CD3ε*) – part of the TCR-CD3 complex – were downregulated about 3-fold (Fig. 3E). The *CD79b* antigen, part of the B lymphocyte antigen receptor, was also downregulated (Fig. 2E). Next, we studied if surface markers involved in T cell activation and survival were differentially expressed in Evi-GOF and control teratomas. The B7 family members CD80 (B7–1) and CD86 (B7–2) work in tandem to activate T cells *via* binding to CD28 co-receptor. Following activation, the inhibitory receptor CTLA-4 is induced in T cells, which compete with CD28 for CD80 and CD86 ligands and provided an inhibitory signal to stop T cell activation. In contrast to *CD86*, *CD80* expression was reduced in Evi-GOF teratomas suggesting an imbalance in T cell activation in Evi-GOF tumors compared to controls (Fig. 3F). However, *CTLA-4*, *PD-L1* and *MHC* class *II* and *I* markers were not significantly changed, suggesting that no major differences regarding tumor recognition by the immune system were apparent (Fig. 3F). Imunohistochemical analysis against MHC class II revealed heterogeneous but similar staining pattern (Fig. 3G). This result might be due to the fact, that embryonic stem cells are non-cancer cells but develop benign teratomas composed of cells resembling normal derivatives and therefore, other than tumor cells, do not use mechanisms to become less immunogenic. Further detailed quantitative analysis on marker gene expression would be necessary to confirm this conclusion.

2.3. Evi overexpression affects teratoma-immune-cell crosstalk

Expression studies showed that Evi-overexpressing ESCs favored a cardiomyocyte gene expression profile, and that immune regulatory genes were downregulated. Enhanced muscle differentiation might be associated with an altered immune response indicating that the cardiomyocyte lineage itself and not the Wnt overexpressing teratoma cells in general is linked to reduced immune cell crosstalk. Therefore, we compared the expression of the cardiomyocyte marker *troponin C type 1* (*Tnnc1*) and the skeletal muscle marker *myosin heavy chain 8* (*Myh8*) as a surrogate marker of cardiomyocyte differentiation, with the expression pattern of the immune cell markers *CD3* (T cells) and *CD19* (B cells). The correlation (R^2) between *Myh8* and *Tnnc1* with *CD3* or