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## Gpr171, a putative P2Y-like receptor, negatively regulates myeloid differentiation in murine hematopoietic progenitors

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### Abstract

Gpr171 is an orphan G protein-coupled receptor putatively related to the P2Y family of purinergic receptors (P2YRs) for extracellular nucleotides, a group of mediators previously shown to regulate hematopoietic progenitor cells (HPCs). No information is currently available on the ligand responsible for Gpr171 activation and its biological role remains unknown. We reconstructed Gpr171 phylogenesis in mice and confirmed that Gpr171 is evolutionally related to members of a P2Y gene-cluster localized on mouse chromosome 3. As a first step towards unveiling a role for Gpr171, we investigated its expression profile in murine hematopoietic cells. As opposed to other P2YRs, we found that *Gpr171* expression is downregulated in monocytes and granulocytes, suggesting a negative role in myeloid lineage-specification. To test Gpr171 functional role, we next enforced *Gpr171* expression in a myeloblastic cell line (32D cells) and in primary Sca-1<sup>+</sup> hematopoietic progenitors (HPCs), and observed a decreased expression of myeloid markers upon induction of *Gpr171*, as well as an increased generation of colonies in vitro. Conversely, *Gpr171* silencing induced opposite results, diminishing the expression of myeloid markers and the clonogenic potential of 32D cells. In vivo, mice transplanted with HPCs over-expressing Gpr171 displayed a significant reduction in the percentage of Mac-1<sup>+</sup>Gr-1<sup>-</sup> cells. As a preliminary step in the investigation of Gpr171 role in murine hematopoiesis, our findings indicate that the orphan receptor Gpr171 negatively regulates myeloid differentiation. Together with phylogenetic analyses, our data suggest that *Gpr171* may have followed a separate evolutionary pathway as compared to other P2YRs belonging to the same gene-cluster.

### Keywords

Gpr171; GPRs; P2 receptors; myeloid differentiation

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#### Author Disclosure Statement

No competing financial interests exist.

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## Introduction

Lifelong hematopoiesis is built on the fine balance between the capability of hematopoietic stem cells to undergo either self-renewal or multi-lineage differentiation [1]. Despite intense investigation, the molecular mechanisms underlying the multi-step process that leads from committed progenitors to terminally differentiated cells are still poorly understood. Over the past years, a few transcription factors have been identified as master regulators of lineage specification [2–4], such as *Gata2* [5, 6], indispensable for megakaryocyte/erythrocyte development. Beside the critical role played by these factors in driving differentiation, transcriptional programs are also modulated by environmental factors through the activity of membrane receptors, such as G protein-coupled receptors (GPRs). GPRs, also known as seven-spanning membrane receptors, represent one of the largest receptor families in nature [7]. They are involved in the molecular decoding of extracellular signals generated by a wide variety of chemically diverse compounds, including small molecules, as well as peptides and proteins. Because of their role in communication between cells and the extracellular environment, seven-spanning membrane receptors have been the focus of intense investigation, aimed to identify their functional role and potential involvement in diseases [8]. GPR ligands comprise growth factors, cytokines, and chemokines, thus making GPRs one of the most investigated pharmaceutical targets [9, 10].

First identified as a putative member of the GPR family, Gpr171 (initially named H963) still remains an orphan receptor, and its ligand, signaling pathway and function are yet to be identified [11]. Based on sequence homology, Gpr171 has been suggested to belong to a particular subfamily of GPRs, named P2Y receptors, which are activated by extracellular nucleotides [12]. Beside the widely acknowledged role of triphosphate-nucleotides in cell metabolism, these molecules are also ubiquitous regulators of a great variety of biologic functions, comprising cell proliferation, differentiation, as well as migration and cell death [13, 14]. For instance, extracellular nucleotides and their cognate receptors have been implicated in the regulation of hematopoietic stem/progenitor cells, as well as in the function of terminally differentiated elements in the peripheral blood (PB) [15–17]. Usually compartmentalized inside the cytoplasm, upon mechanical, chemical or infective stress nucleotides can be released into the extracellular microenvironment [18], where they interact with purinergic membrane receptors [19–21]. Purinergic receptors comprise two subfamilies, classified as membrane channel-receptors (P2X) and 7-spanning receptors (P2Y receptors or P2YRs) [22]. As members of the GPR family, P2YRs transduction of intracellular signals proceeds through coupling to G proteins [19].  $G_{q/11}$  represents the G protein mainly activated by P2YRs, although in the case of P2Y12, P2Y13, and P2Y14  $G_{i/o}$  proteins have been shown to be recruited [23].

The data recently provided by the sequencing of mammalian genomes permitted the identification of new members of this family. The present study represents a preliminary attempt to investigate the role of Gpr171 in hematopoietic progenitor cells (HPCs). Our findings indicate a peculiar evolutionary history for Gpr171 as compared to other members of the P2Y family and suggest that Gpr171 may act as a negative regulator of myeloid differentiation, both in vitro and in vivo.

## Materials and Methods

### Sequence analyses

Sequence alignments and phylogenetic analyses were performed using the program CLC MainWorkbench 5.5. Phylogenetic trees were created using the Neighbor-Joining (NJ) method and 1,000 bootstraps replications were conducted to evaluate the reliability of the trees. The putative transmembrane domains were predicted using the program TMPred

([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) [24]. The following accession numbers were entered for alignment analyses of amino acid sequences: *Gpr87*\_Mouse (NP\_115775), *Gpr99*\_Mouse (NP\_001001490), *Gpr171*\_Mouse (NP\_775574), *P2ry1*\_Mouse (NP\_032798), *P2ry2*\_Mouse (NP\_032799), *P2ry4*\_Mouse (NP\_065646), *P2ry6*\_Mouse (NP\_898991), *P2ry12*\_Mouse (NP\_081847), *P2ry13*\_Mouse (NP\_083084), *P2ry14*\_Mouse (NP\_573463), *Sucnr1*\_Mouse (NP\_115776).

### Cell line and mice

The hematopoietic progenitor cell line 32D was obtained from ATCC and maintained in RPMI 1640 medium, supplemented with 10% FBS and rIL-3 (5ng/mL). For studies *in vivo*, wild type (WT) C57BL/6, CD45.1 mice (6 to 12 weeks of age) were utilized for this study. Animals were housed and bred in specific pathogen-free conditions at Baylor College of Medicine.

### Construction of retroviral vectors

The plasmids containing the genes described in the present study were purchased from ATCC and subsequently subcloned into the retroviral expression vector MSCV-IRES-eGFP. Retroviral particles were packaged by co-transfection of 293t cells with MSCV-GOI-IRES-eGFP and pCL-Eco plasmids as previously described [25]. 48 hours after transfection, culture supernatants were collected and their viral titer was determined by infecting NIH3T3 cells. As a control, the mock construct MSCV-IRES-eGFP was used. In 32D cells, *Gpr171* expression was induced by direct nucleofection according to manufacturer's instructions (Amaxa Cell Line Nucleofector Kit C, Lonza).

### Retroviral transduction of bone marrow progenitor cells and transplantation

The enforced expression of *Gpr171* was assayed by retroviral transduction of WT HPCs. WT donor mice (C57BL/6, CD45.2, 6–12 weeks of age) were i.p. injected with 5-Fluorouracil at 150 mg/Kg. Six days later, BM cells were harvested from tibias, femurs, and hips and enriched by immuno-magnetic separation (AutoMACS, Miltenyi Biotech, Auburn, CA) for Sca-1<sup>+</sup> HPCs. Sca-1<sup>+</sup> cells were seeded in 24-well plates (500,000 cells/well) in StemPro medium (GIBCO, Carlsbad, CA), supplemented with nutrient supplement 40X (GIBCO), L-Glutamine, Penicillin/Streptomycin, Polybrene (4 µg/mL), rmTPO (R&D, Minneapolis, MN), and rmSCF (R&D) and spin-infected (at 1,100 rpm for 2 hours, at room temperature) in the presence of retroviral vectors (MSCV-IRES-eGFP as a control, or MSCV-*Gpr171*-IRES-eGFP). After transduction, cells were harvested and transplanted into lethally irradiated C57BL/6, CD45.1 recipient mice (50,000 – 75,000 cells/mouse). PB engraftment and lineage distribution of CD45.2 GFP<sup>+</sup> cells were analyzed at 5, 12, 22 and 28 weeks after transplantation.

### Gpr171 silencing

A total of 10<sup>6</sup> 32D cells were washed twice in PBS and resuspended in nucleofector solution (Amaxa Cell Line Nucleofector Kit C, Lonza) and mixed with siRNAs specific for *Gpr171* silencing (SASI\_Mm02\_00345246 and SASI\_Mm02\_00345246\_AS, Sigma-Aldrich). Cells were treated with Nucleofector program E-32, according to manufacturer's instructions. Nucleofected cells were immediately removed from the cuvette by adding pre-warmed RPMI + 10% FBS medium and incubated at 37°C, 5% CO<sub>2</sub>. After 48 hours, cells were used for real-time PCR analysis, CFU-C *in vitro* assays and phenotypic analysis.

### Real-time PCR analysis

Total RNA was isolated from treated cells using RNeasy Micro Plus Kit (Qiagen) First strand synthesis was performed with Improm2 (Promega) and real-time PCR was performed

with Taqman probe sets (Applied Biosystems) on a ABI Prism 7700 Sequence Detector for 40 cycles. A mouse internal control (*Gusb*) was included in every reaction for normalization and expression was measured for each assay relative to the *Gusb* internal standard ( $\Delta\text{Ct}$ ). The fold change was calculated from the formula  $2^{\Delta\Delta\text{Ct}}$ . The error bars represent the corresponding SEM.

### In vitro Colony-Forming-Unit (CFU-C) assay

For CFU-C assays, primary Sca-1<sup>+</sup> cells were cultured at 37°C, 5%CO<sub>2</sub> for 48 hours. GFP<sup>+</sup>Sca-1<sup>+</sup> cells were then sorted (on a MoFlo flow cytometer) into 96-well plates (1 cell/well), containing M3434 MethoCult medium (Stem Cell Technologies, Vancouver, Canada), and incubated at 37°C, 5%CO<sub>2</sub>. After 12–14 days of culture, hematopoietic colonies were counted.

### Flow-cytometry

32D cells or primary Sca-1<sup>+</sup> cells were transduced with retroviral vectors as described, maintained in liquid culture for at least 48hrs and then stained with the following antibodies for phenotypic analysis: anti-Mac1 PE-Cy7 or APC, anti-CD16/32 PE, anti-Gr-1 APC or PE, biotinylated anti-F4/80 (followed by anti-biotin Pacific Blue, PB), and anti-CD14 PE. PB from transplanted mice was collected from the retro-orbital sinus at 5, 12, 22 and 28 weeks after transplantation. As previously described [26], the samples were pre-treated with Red Blood Cell – lysis buffer (TrisCl pH 7.8 and Ammonium Chloride, 1:9) and then stained for 20 minutes on ice with monoclonal antibodies, according to the following cocktail: anti-CD45.2 APC-conjugated, anti-CD4 PB, anti-CD8 PB, anti-B220 PB, anti-B220 PE-Cy7, anti-Mac1 PE-Cy7, and anti-Gr1 PE. All samples were resuspended in Hanks' buffer containing Propidium Iodide (PI, 1 µg/mL), and analyzed on a LSRII cytofluorimeter (BD bioscience, Franklin Lakes, NJ).

### Statistics

Results of experimental data are reported as mean ± SEM. Statistical significance was determined by two-tailed Student's *t* test. P values are indicated as \**p*<0.05 and \*\**p*<0.01.

## Results

### Gpr171 is evolutionally related to P2YRs and its expression is suppressed upon myeloid differentiation

Based on sequence homology, it has been suggested that human GPR171 is evolutionally and structurally related to the GPR subfamily of purinergic P2YRs for extracellular nucleotides [27]. Similarly to the human gene, when we aligned the amino acid sequences of murine Gpr171 and other murine P2YRs, we found a high degree of similarity, especially in correspondence of the sequences encoding for transmembrane domains (TM), particularly TM3, TM6, and TM7 (Fig. 1A). By reconstructing the phylogenetic tree of P2YRs encoded by the murine genome, we also found that the murine *Gpr171* is evolutionally related to P2Y genes, particularly *P2ry12*, *P2ry13*, *P2ry14*, and *Gpr87* (Fig. 1B). Interestingly, these five genes co-localize on chromosome 3, as depicted in Fig. 1C. Since co-localization of genes in the same chromosomal region is a strong indication of local gene amplification, the clustering of *Gpr171*, *P2ry14*, *Gpr87*, *P2ry13*, and *P2ry12* is highly suggestive of events of gene duplication, followed by divergent evolution. Reconstructing the evolution of orphan receptors, such as Gpr171, could also help identify its cognate ligand, since receptors evolved from a common ancestor might share, at least partly, ligand-specificity.

In an attempt to identify a functional role for Gpr171, we first consulted a database previously generated in our laboratory, comprising the genetic expression profile of HSCs,

as well as their differentiated progeny [25]. By bio-informatic analysis, genes that are selectively expressed in the HSC compartment were discriminated from genes uniquely involved in the specification of the differentiated lineages (including granulocytes, macrophages, erythrocytes, NK cells, B and T lymphocytes). Using this approach specific lineage “fingerprints” were defined, comprising a combination of genes uniquely expressed in a particular hematopoietic population [25]. From the comparison of the expression profile of HSCs and their differentiated progeny, *Gpr171* emerged as a gene consistently expressed in the lymphoid lineage (activated and naïve T Lymphocytes, B Lymphocytes, and NK cells), as well as in the HSC compartment (identified as Side Population c-Kit<sup>+</sup> Lineage<sup>-</sup> Sca-1<sup>+</sup> cells, SP<sup>KLS</sup> cells [25, 26]). In contrast, *Gpr171* expression appeared to be drastically suppressed in cells that underwent myeloid differentiation (monocytes, granulocytes, and erythrocytes; Fig. 2, top panel). Interestingly, when we analyzed the expression profile of genes highly related to *Gpr171*, such as *P2ry12*, *P2ry13*, and *P2ry14*, we found a strikingly different pattern. As opposed to *Gpr171*, all these genes appear to be consistently up-regulated in cells belonging to the myeloid lineage and down-regulated in activated T cells, as well as B cells (Fig. 2). These findings suggest that an early bifurcation in the phylogenetic tree might have occurred, originating, on one side, *Gpr171* and, on the other, *P2ry12*, *P2ry13*, *P2ry14*, and *Gpr87* (Fig. 1B). The divergent evolution resulting from this bifurcation could have contributed to specializing *Gpr171* in functions different from those described for the genes originating from the sibling branch.

### **Gpr171 is silenced in differentiating 32D cells and its enforced expression down-regulates myeloid differentiation**

Data mining is an exceptional source of information about unknown, orphan receptors. Nonetheless, the ultimate validation of data provided by bio-informatic analysis is based on functional studies. Gene expression profiling in the hematopoietic system suggested a negative correlation between *Gpr171* and myeloid differentiation (Fig. 2). In order to test this hypothesis, we first studied how *Gpr171* expression was modulated during differentiation in an immortalized monoblast-like cell line (32Dcl.3 cells, henceforth referred to as 32D cells), widely used as a model for hematopoietic progenitor cells (HPCs). 32D cells proliferate as undifferentiated blasts when maintained in culture medium containing IL-3. On the contrary, IL-3 withdrawal and G-CSF treatment have been demonstrated to promote granulocytic differentiation in these cells [28]. To assess the relationship between *Gpr171* and myeloid differentiation, 32D cells were cultured in the presence of G-CSF and in the absence of IL-3. As expected, differentiating 32D cells presented after three days an increase of in surface myeloid markers (such as Mac-1 and Gr-1, data not shown). In addition, G-CSF-treated cells presented a significant increase in the relative expression of two genes involved in myeloid differentiation, namely the colony stimulating factor 1-receptor *Csf1r* and the myeloperoxidase *Mpo*, whereas *Gpr171* expression was almost completely silenced (Fig. 3A). In order to confirm the negative relationship between myeloid differentiation and *Gpr171*, we enforced *Gpr171* expression in 32D cells using a vector derived from Mouse Stem Cell Virus (MSCV) (as shown in Fig. 3B). Phenotypic analyses of markers typically associated with myeloid differentiation showed that both the expression and the mean fluorescence intensity (MFI) of Mac-1, Gr-1, F4/80, CD16/32, and CD14 appeared to be markedly decreased upon over-expression of *Gpr171* in 32D cells (Fig. 3C–D). In addition, we observed that the enforced expression of *Gpr171* in 32D cells increases cell proliferation in liquid culture (Fig. 3E), as well as colony formation (Fig. 3F).

In order to assess the capability of *Gpr171* over-expression to counteract myeloid differentiation, we enforced *Gpr171* expression and cultured transfected 32D cells in the absence of IL-3: a condition known to hinder self-renewal and promote cell differentiation.

After 8 days, the percentage of Mac-1 positive cells in the *Gpr171* over-expressing cell population was 20% less than the control, suggesting that even under conditions that allow differentiation of 32D cells the enforced expression of *Gpr171* decelerates myeloid commitment (Fig. 3G). Taken together, these initial experiments suggest that *Gpr171* may play a role in counteracting myeloid commitment by inhibiting cell growth and down-modulating the expression of myeloid markers in a cell line model of myeloblastic precursors.

### **Gpr171 silencing promotes myeloid differentiation in 32D cells**

To further test the role of *Gpr171* in myelopoiesis, we transduced 32D cells with a mix of siRNAs specifically designed to silence *Gpr171* expression. After 48 hours, we found a 52% reduction in *Gpr171* expression in transduced 32D cells. Concomitantly, treated cells presented an increase in the relative expression of *Csf1r* and *Mpo*, two genes linked to myeloid differentiation (Fig. 4A). In addition, the membrane expression of both Mac-1 was increased in 32D cells transduced with anti-*Gpr171* siRNAs, as assessed by flow cytometry (Fig. 4B–C). Similarly, the mean fluorescence intensity for Gr-1 was also increased in 32D cells transduced with anti-*Gpr171* siRNAs (Fig. 4D). In contrast, *Gpr171* over-expression induced the opposite effect, down-regulating Mac-1 expression in transduced cells (Fig. 4B–C).

Functionally, the up-regulation of differentiation markers was paralleled by a decreased proliferative capacity of *Gpr171*-silenced 32D cells (38% reduction compared to the control), as displayed by *in vitro* clonogenic assays (Fig. 4E). Conversely, when *Gpr171* over-expression was induced in 32D cells, a 20% increase in CFU-C numbers was recorded (Fig. 4E). Taken together, these findings contribute to define the role of *Gpr171* in hematopoiesis and suggest that the expression of *Gpr171* negatively regulates myeloid differentiation.

### **Gpr171 enforced expression in primary mouse HPCs inhibits the expression of myeloid markers both in vitro and in vivo**

In order to test the effects of *Gpr171* over-expression in primary murine cells, we retrovirally transduced Sca-1 – enriched HPCs, according to the experimental model depicted in Fig. 5A. Similarly to what we observed in 32D cells, *Gpr171* over-expression in Sca-1<sup>+</sup> progenitors suppressed the membrane expression of myeloid markers, such as Mac-1, Gr-1, F4/80, CD16/32, and CD14 (Fig. 5B–C).

In order to investigate how powerfully *Gpr171* can inhibit myeloid differentiation, we retrovirally transduced Sca-1<sup>+</sup> HPCs and transplanted them into lethally irradiated mice. PB reconstitution was assessed by flow cytometry at different time points (5, 12, 22, and 28 weeks after transplant), according to the model depicted in Fig. 6A (left panel). Transduction efficiency, as inferred from the percentage of donor-derived CD45.2 GFP<sup>+</sup> cells, was about the same at 5 weeks after transplantation in both control and experimental mice (21.18% vs 17.19% for MSCV-*Gpr171* and MSCV control, respectively, data not shown).

Next, we analyzed how *Gpr171* enforced expression affects the contribution to the different hematopoietic lineages *in vivo* (Fig. 6A). At all the time points considered, we observed no significant skewing in the percentages of myeloid cells (Mac-1<sup>+</sup> cells), B lymphocytes (B220<sup>+</sup> cells), and T lymphocytes (CD4<sup>+</sup>CD8<sup>+</sup> cells) in the subset of donor-derived, GFP<sup>+</sup> cells (Fig. 6B). However, when we dissected the myeloid compartment on the basis of Mac-1 and Gr-1 expression, we found that the monocytic population (Mac-1<sup>+</sup>Gr-1<sup>-</sup>) was consistently reduced upon *Gpr171* over-expression (Fig. 6C–D). At 5 weeks post-

transplantation, we observed a 1.4-fold decrease in the percentage of GFP<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>-</sup> cells in the Gpr171-group, compared to the control (Fig. 6D). This divergence became even more evident at 12 weeks after transplantation (when GFP<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>-</sup> cells displayed a 1.7-fold decrease upon *Gpr171* over-expression) and was maintained up to 28 weeks post transplantation (Fig. 5D). This findings confirm that Gpr171 plays a role in lineage specification and it is capable of inhibiting monocyte differentiation *in vivo*, as well as *in vitro*.

## Discussion

Gpr171 is a novel, putative GPR member, structurally and evolutionally related to P2YRs. Since Gpr171 was first described, no progress has been made in deciphering its biological role and its physiological ligand [11]. In this report, we investigated Gpr171 function in the murine hematopoietic system and found that its expression negatively correlates with myeloid differentiation, both *in vitro* and *in vivo*. In addition, despite the similarity that Gpr171 shares with P2YRs, our data contribute to highlight a negative role for Gpr171 in myelopoiesis, as opposed to other members of the family highly expressed in granulocytes and monocytes.

Recently, Simon and Barnard found in humans a striking similarity between GPR171 (previously known as H963) and some members of the family of human P2YRs for extracellular nucleotides [27]. By mapping the exact chromosomal localization of all P2YRs so far identified, the authors found five orphan GPCRs (among which was GPR171), that are localized in the same region of chromosome 3, where the human genes for GPR87, P2Y12, P2Y13, and P2Y14 cluster together. Furthermore, the GPR171 sequence shares specific motifs in the transmembrane domains TM6 and TM7, found to be highly conserved among P2YRs. Based on the evidence provided by evolutionary and structural similarity, the orphan receptor Gpr171 likely represents a new member of the P2Y receptor-family, involved in the transduction of signals generated by extracellular nucleotides. Despite the phylogenetic relationship between Gpr171 and other members of the P2Y family, our results in the murine system suggest that Gpr171 might have followed a divergent evolutionary pathway, causing a substantial deviation in terms of biological function. We showed that *P2ry12*, *P2ry13*, and *P2ry14*, although part of the same P2Y cluster on chromosome 3, display the opposite expression pattern of *Gpr171*. Indeed, even minor mutations in the amino acid sequence of a receptor can give rise to significant evolutionary detours, leading to a different agonist specificity or signaling pathway. Hence, unveiling the physiological agonist for Gpr171, as well as developing specific antagonists, will help achieve a deeper understanding of the pharmacologic behavior of this, so far, orphan receptor and of its relationship to the other members of the family.

In this study, we investigated the expression profile of *Gpr171* in hematopoietic cells (including the compartment of SP<sup>KLS</sup> HSCs) and found that Gpr171 is significantly suppressed in cells undergoing myeloid differentiation. In contrast, other P2YRs belonging to the same gene-cluster (e.g. P2y12, P2y13, and P2y14) present the opposite pattern of expression, with the highest levels recorded in differentiated myeloid cells (Fig. 2). Such a difference might be the result of an early bifurcation in the phylogenetic tree leading to the divergent evolution of Gpr171 and other genes of the same cluster on Chromosome 3. At the functional level, the enforced expression of Gpr171 in both 32D cells and primary Sca-1<sup>+</sup> HPCs decreased the membrane expression of monocyte-specific antigens, such as Mac-1, CD14, F4/80, and CD16/32. In line with these findings, *Gpr171* silencing induced opposite results, diminishing the expression of myeloid markers and the clonogenic potential of 32D cells. *In vivo*, when Sca-1<sup>+</sup> HPCs transduced with MSCV-*Gpr171* retrovirus were transplanted into lethally irradiated mice, a specific decrease in the percentage of

Mac-1<sup>+</sup>Gr-1<sup>-</sup> monocytes was consistently observed in the myeloid fraction of *Gpr171* over-expressing mice over a period of 28 weeks post-transplantation.

Taken together, these findings support the hypothesis that the orphan receptor Gpr171 negatively regulates myeloid differentiation and, together with phylogenetic analyses, suggest that Gpr171 may have followed a separate evolutionary pathway as compared to other P2Y receptors of the same chromosomal cluster.

In the future, a deeper understanding of the role of Gpr171 in murine hematopoiesis is expected to arise from additional in vivo studies. The definitive determination of the role of Gpr171 in the hematopoietic system will involve the characterization of Gpr171 knockout mice, as well as the repression of *Gpr171* expression in hematopoietic reconstitution assays. In addition, future investigations of the biological role of Gpr171 will greatly benefit from the identification of its physiological ligand. Based on the structural similarity that Gpr171 shares with P2Y receptors, we expect that its ligand may belong to the family of extracellular nucleotides. This group of molecules has recently emerged to play a role in immunity, inflammation, as well as in the regulation of the hematopoietic system both in animal models and humans [15–17, 29]. Over the past years, the differentiation of myeloid, lymphoid, mekacaryocytic, and erythroid progenitors has been shown to be influenced by extracellular nucleotides through the activation of purinergic receptors [30]. Interestingly, depending on the specific hematopoietic lineage, different P2 receptors appear to play a role in differentiation and the identification of novel orphan receptors will contribute to a deeper understanding of hematopoietic differentiation. The results emerged from our preliminary investigation show that Gpr171, as a putative novel member of the P2Y family, may be involved in the differentiation and proliferation of myeloid cells, suggesting a novel mechanism by which extracellular nucleotides can tune immunological responses [31].

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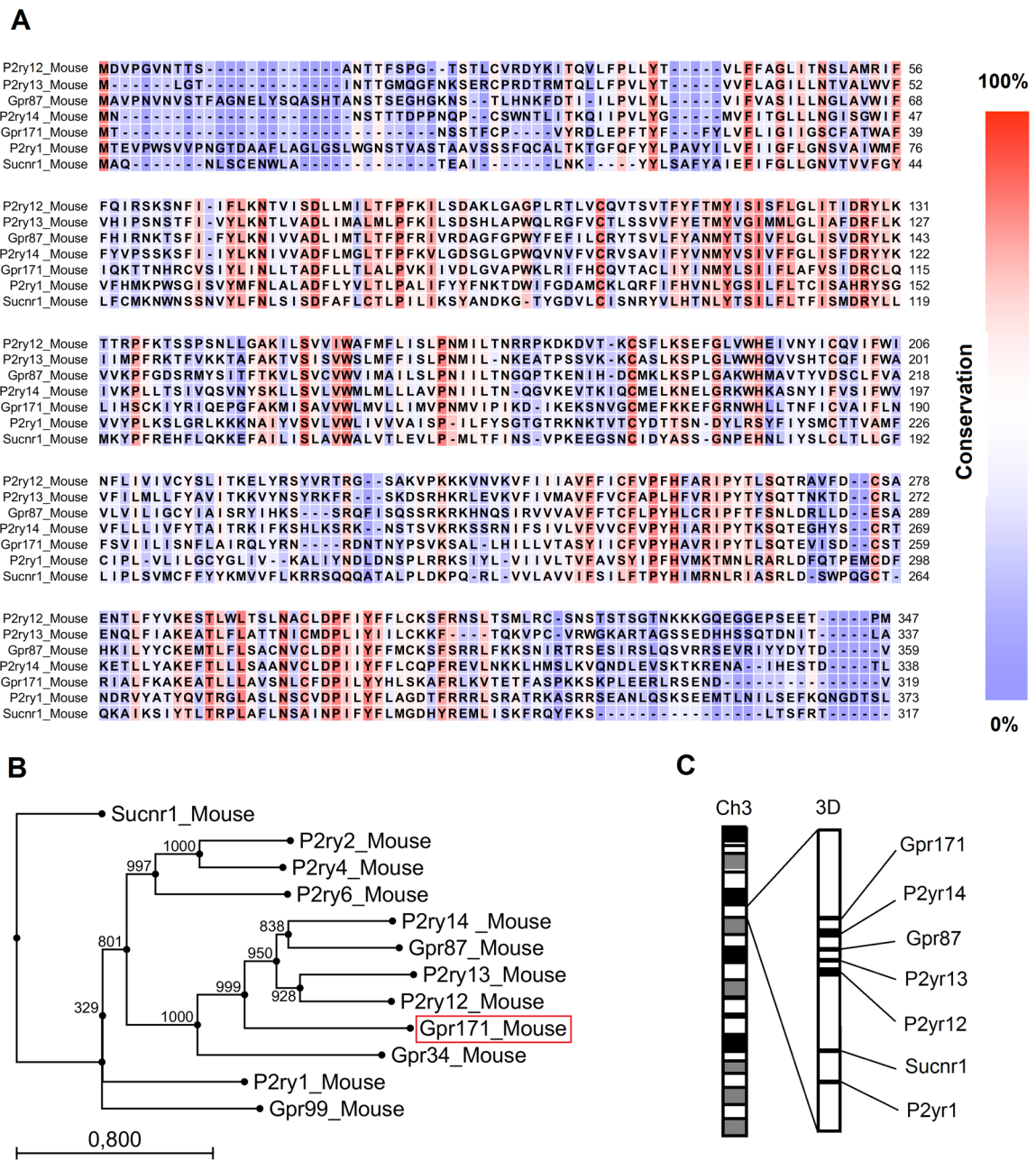
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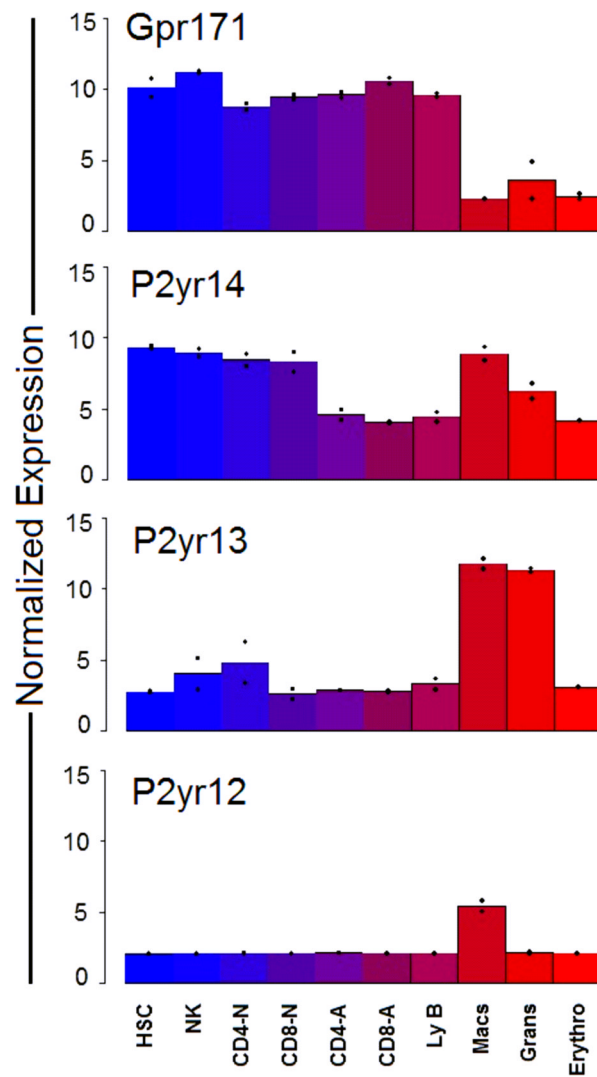
**FIG. 1.** Phylogenetic analysis of Gpr171. **(A)** Alignment of amino acid sequences of murine Gpr171 with other murine P2Y genes localized on chromosome 3. The seven transmembrane domains (indicated as TM) are overlined. As emphasized by the color scheme, the sequences display the highest degree of conservation in their TM3, TM6, and TM7 domains. **(B)** Phylogenetic tree illustrating how the murine orphan receptor Gpr171 is related to other members of the P2YR family. Alignment of amino acid sequences and tree reconstruction were performed using CLC Main Workbench 5. Trees were created using the Neighbor-Joining (NJ) method and 1,000 bootstraps replications were conducted. The length of each branch is proportional to evolutionary divergence. **(C)** Schematic representation of the reciprocal localization of Gpr171, Gpr87, P2ry12, P2ry13, P2y14, Sucnr1, and P2ry1 on

chromosome 3. Co-localization of genes in the same chromosomal region strongly indicates that they evolved by gene duplication from the same ancestor gene.

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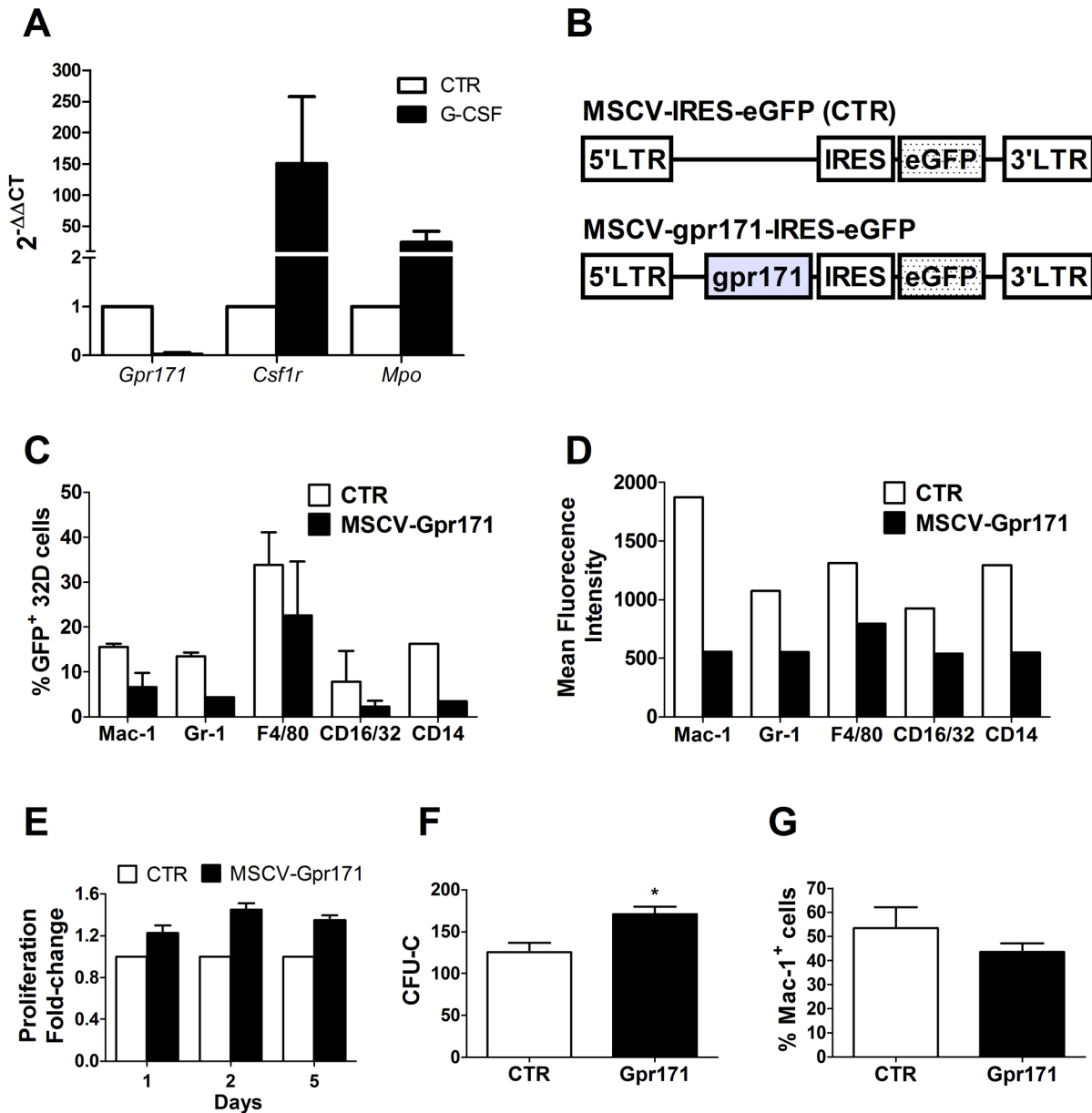
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**FIG. 2.**

Expression levels of Gpr171, P2ry14, P2ry13, and P2y12 in murine hematopoietic cells. The normalized ( $\log_2$ ) expression intensity of Gpr171 expression (top panel) in myeloid cells (macrophages, granulocytes, and erythrocytes) is significantly decreased compared to the expression of other members of the same P2ry cluster on chromosome 3 (P2ry14, P2ry13, and P2y12).



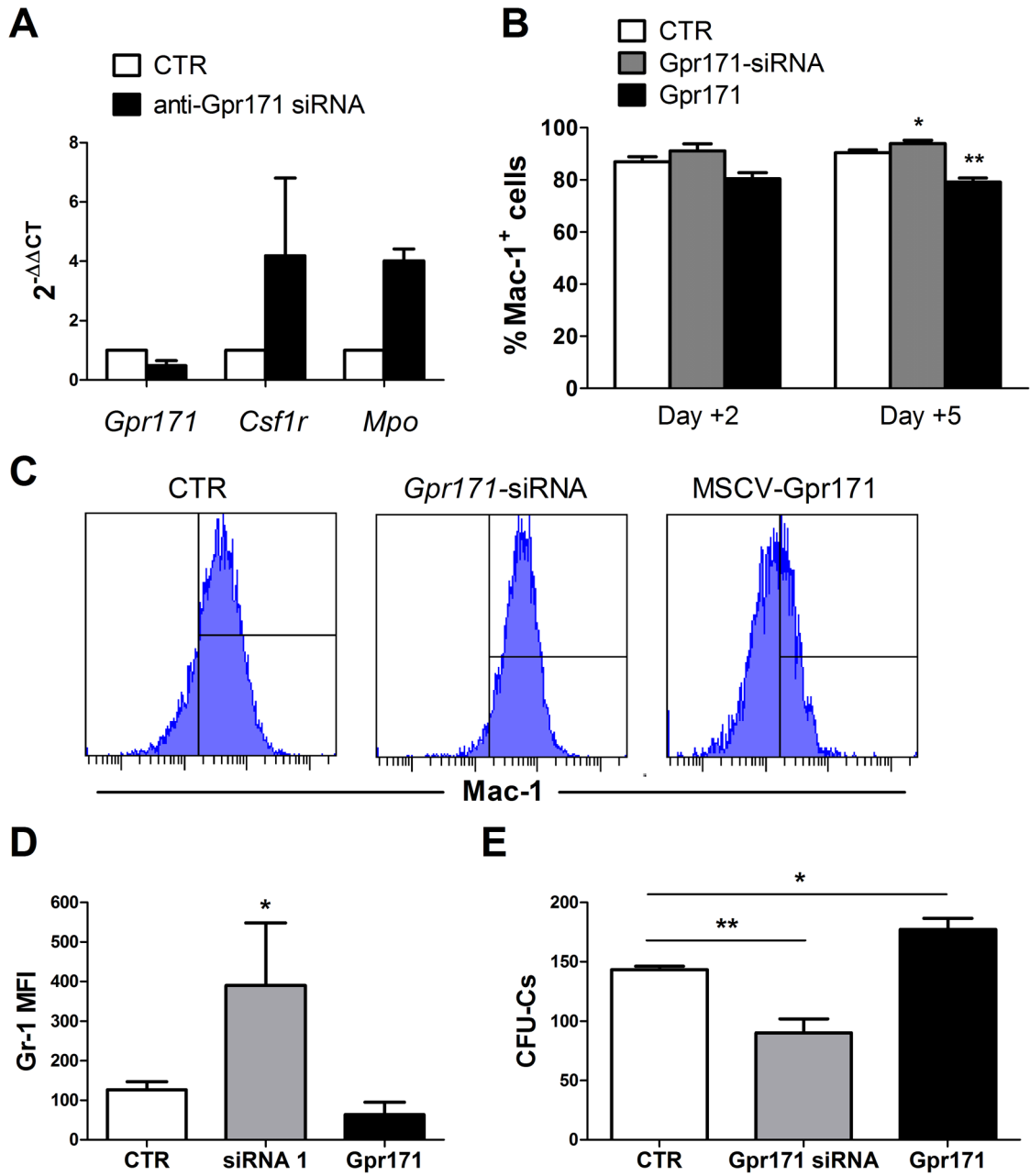
**FIG. 3.** Gpr171 expression in 32D cells. **(A)** Relative expression of *Gpr171*, *Csf1r*, and *Mpo* in 32D cells after 3 days of culture under differentiating conditions (in the presence of G-CSF). **(B)** Schematic representation of the MSCV retroviral expression vector utilized to transduce hematopoietic cells. The mock construct (top) expresses the reporter gene GFP, while the Gpr171 construct (bottom) allows the coordinated expression of both Gpr171 and GFP. **(C)** Phenotypic characterization of transduced 32D cells: percentage of 32D cells expressing myeloid markers are decreased after induction of Gpr171 expression. **(D)** Mean Fluorescence Intensity (MFI) of myeloid markers in 32D cells over-expressing Gpr171. **(E)** Proliferation is increased in Gpr171-overexpressing 32D cells, as emerged over a 5-days time-course. Data are expressed as proliferation fold-change between MSCV-Gpr171 transduced cells and control cells. **(F)** Colony formation (expressed as CFU-Cs) is increased in 32D overexpressing Gpr171. **(G)** Modulation of Mac-1 expression under conditions

driving myeloid differentiation in 32D cells. Transduced 32D cells were cultured for 8 days in the absence of IL-3. Results are reported as mean  $\pm$  SEM of at least triplicate experiments. \* $p < .05$ , \*\* $p < .01$ .

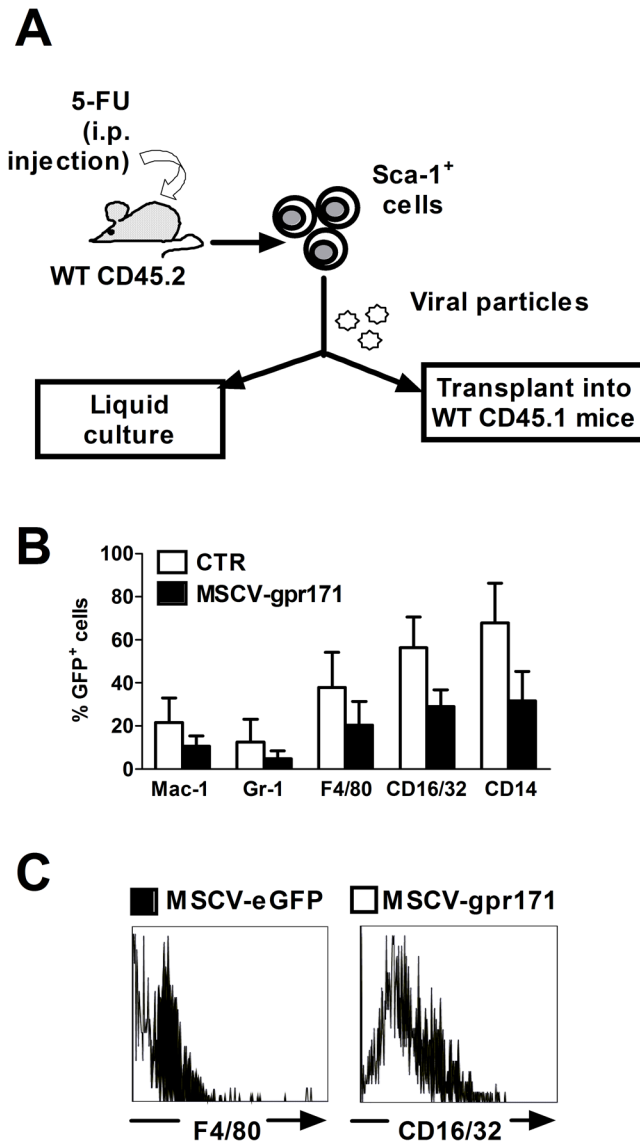
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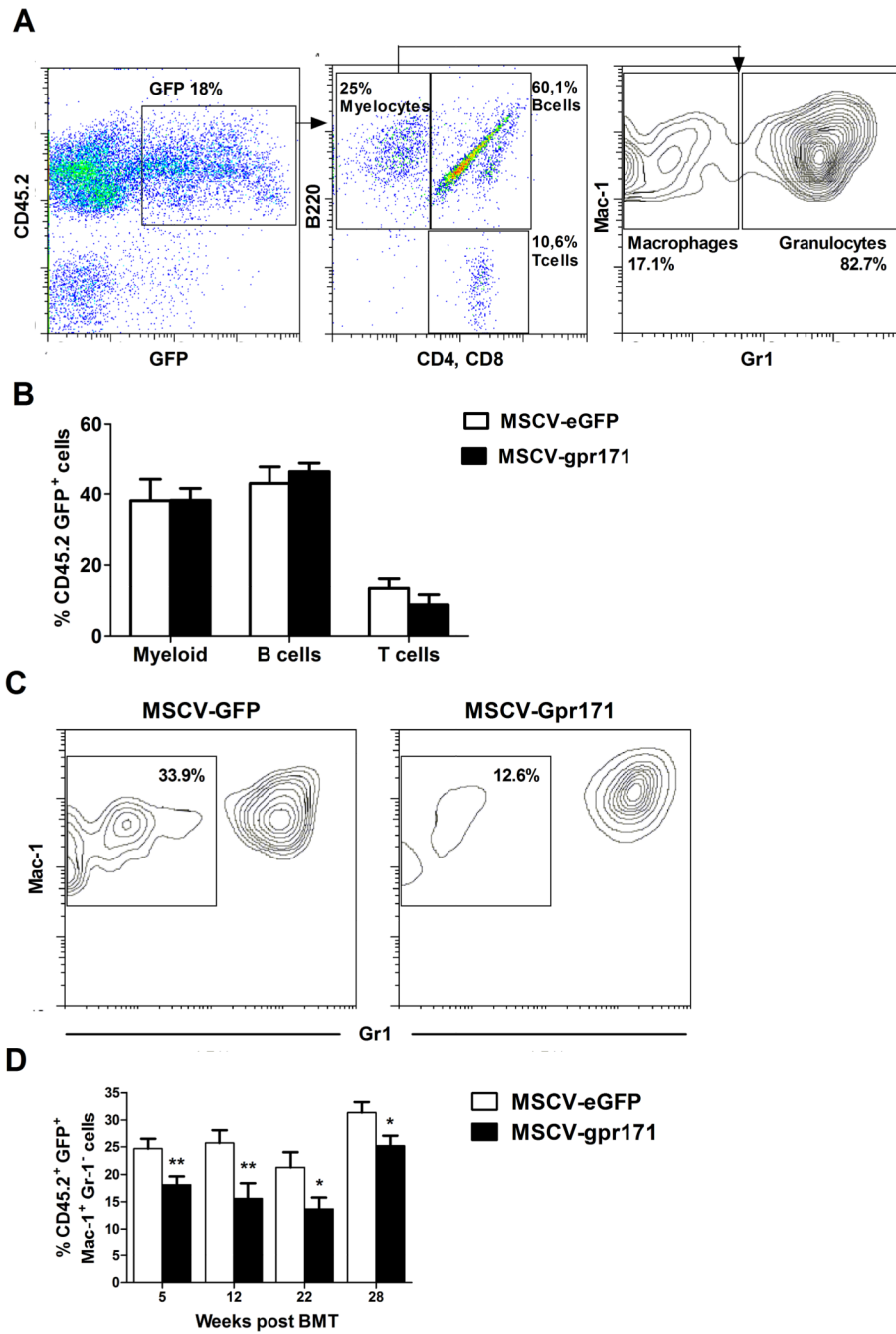


**FIG. 4.** (A) Relative gene expression of *Gpr171* and myeloid differentiation genes (*Csf1r* and *Mpo*) in 32D cells transduced with anti-*Gpr171* siRNAs. Cells were analyzed 48hrs post-transduction and mRNA levels were assessed by Real-Time PCR. (B) Percentages of Mac-1<sup>+</sup> 32D cells after transduction with MSCV-*Gpr171* or anti-*Gpr171* siRNAs. Cells were analyzed by flow-cytometry 2 and 5 days post-transduction. (C) Representative histograms of Mac-1 expression in 32D cells 96 hours after transduction with MSCV-*Gpr171* or anti-*Gpr171* siRNAs. (D) Mean Fluorescence Intensity (MFI) of myeloid marker Gr-1 in 32D cells transduced with MSCV-*Gpr171* or anti-*Gpr171* siRNAs. (E) 32D colony formation (expressed as CFU-Cs) is decreased after transduction with anti-*Gpr171* siRNAs.



**FIG. 5.** Gpr171 over-expression in Sca-1<sup>+</sup> cells *in vitro*. (A) Schematic representation of the experimental model utilized to study Gpr171 role in murine hematopoiesis. Sca-1<sup>+</sup> HSCs/HPCs were collected from WT CD45.2 donor mice previously injected with 5-FU and transduced with the retroviral MSCV-Gpr171 vector or with the empty control vector. Transduced cells were then utilized for both *in vitro* and *in vivo* studies. (B) Percentages of GFP<sup>+</sup>, primary murine hematopoietic cells expressing markers associated with myeloid differentiation are decreased after induction of Gpr171 expression. (C) Representative flow plots showing decreased expression of two markers associated with terminal monocyte differentiation (F4/80 and CD16/32), following Gpr171 over-expression. Results are reported as mean ± SEM of at least triplicate experiments.





**FIG. 6.** Gpr171 over-expression *in vivo*. **(A)** Schematic representation of the PB analysis performed periodically on transplanted animals. First, transduced (GFP<sup>+</sup>), donor-derived (CD45.2<sup>+</sup>) blood cells were gated (top), then cells were divided in Mac-1<sup>+</sup> myeloid cells, B220<sup>+</sup> B lymphocytes, and CD4<sup>+</sup>CD8<sup>+</sup> T lymphocytes (middle). Finally, myeloid cells were separated in monocytes and granulocytes, according to Gr-1 expression (bottom). **(B)** Lineage distribution of Mac-1<sup>+</sup> Myeloid cells, B220<sup>+</sup> B Lymphocytes, and CD4<sup>+</sup>CD8<sup>+</sup> T Lymphocytes in the PB of transplanted mice, as assessed by flow cytometry at 12 weeks post-transplant. **(C)** Representative density plots displaying the relative percentages of PB

GFP<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>-</sup> monocytes and PB GFP<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>+</sup> granulocytes, as assessed by flow cytometry at 5 weeks after transplantation (control group on the left and Gpr171 group on the right). **(D)** Time-course analysis of the percentage of PB GFP<sup>+</sup>Mac-1<sup>+</sup>Gr-1<sup>-</sup> monocytes, as assessed by flow cytometry at 5, 12, 22, and 28 weeks after transplantation. Results are reported as mean ± SEM of at least triplicate experiments. \*p<.05, \*\*p<.01.

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