HEDGEHOG SIGNALING PATHWAYS IN MULTIPLE MYELOMA

I. Dulcamare¹,†, S. Giallongo²,†, N. Vicario²,†, G. Scandura¹, A. Barbato¹, E. La Spina¹, L. Longhitano², D. Cambria¹, T. Zuppelli¹, D. Tibullo², D. Lo Furno², R. Parenti², G. Li Volti², G. A. Palumbo³, F. Di Raimondo¹, A. Romano¹,‡, C. Giallongo³,‡

¹ Division of Hematology, Department of General Surgery and Medical-Surgical Specialties, A.O.U. Policlinico Vittorio Emanuele, University of Catania, Catania, Italy
² Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy
³ Department of Medical and Surgical Sciences and Advanced Technologies G.F. Ingrassia, University of Catania, Catania, Italy

† These authors equally contributed to this work as co-first
‡ These authors equally contributed to this work as co-last

ABSTRACT

Multiple myeloma (MM) is a hematological disease characterized by the uncontrolled proliferation of bone marrow malignant plasma cells. Localization and survival of malignant cells relies on bone marrow niche, in turn determined by the interaction between MM cells and mesenchymal stromal cells (MSCs). Several reports suggest that Hedgehog (Hh) pathway plays an outstanding role in tumor microenvironment maintenance. Hh signaling orchestrates the transformation of the myeloma bone marrow microenvironment supporting the proliferation of malignant plasma cells by affecting NF-kB signaling. To date, different clinical approaches are currently undergoing to evaluate the role of Hh modulators as efficient MM therapy. In this review article, we discuss the recent advances in the understanding of Hh signaling pathway in MM microenvironment.
INTRODUCTION

Multiple myeloma (MM) is a hematological disease characterized by bone marrow malignant plasma cells enhanced proliferation (1), usually resulting into hypercalcemia, renal impairment, anemia and bone pain (2). Myeloma bone disease is a devastating complication of MM observed in more than 80% of patients (3). The pathophysiology characterizing this outcome include a series of complex biochemical and cellular processes involving osteoclasts and osteoblasts activity, orchestrated by osteocytes. These cells act as mechano-sensors mediating the bone remodeling process by secreting cytokines such as Osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL). In the MM context, several studies reported an increased RANKL/OPG ratio resulting into osteoclasts activation and disruption of bone marrow homeostasis (5-9). In physiological conditions, osteocytes inhibit osteoblasts differentiation by blockage of the canonical Wingless-type (Wnt) signaling, mediated by sclerostin and Dickkopf-1 (Dkk-1) secretion (10). Elevated amounts of DKK1 in MM patients correlated with the presence of focal bone lesions (11). Moreover, the bone resorption is enhanced by malignant plasma cells, acting by i) releasing macrophage inflammatory protein-1α and β (MIP-1α-β), ii) inducing mature osteoblasts apoptosis and iii) inhibiting the differentiation of their precursors (12-14). As a result, bone matrix degradation releases the growth factors and cytokines boosting MM cells survival (15). For this reason, targeting the osteocytes-osteoblasts axis may represent a promising strategy counteracting MM progression.

The Hedgehog (Hh) signaling pathway holds a critical role for intercellular communication during the development of many organs, while its aberrant activation has been reported in several cancers (16). Hh signaling mostly relies on primary cilium, a microtubule-based organelle in the surface of vertebrate cells serving as mechano-sensory structure towards microenvironment stimuli (17). Consistently, primary cilium may act as a communication hub during organ and embryonic development, immune response, and tissue homeostasis, eventually triggering different cascade, including Wnt signaling (18).

Hh mammalian proteins have been grouped into three classes: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh), in turn explicating different duties within the cellular context. The latter has been reported to play a major role in endochondral ossification during skeletal development, while Dhh expression has been described in pre-Sertoli cells leading male sexual differentiation, and Shh is secreted to mediate epithelial invagination, limbs patterning and nervous system commitment (19-21). Interestingly, activation of the Hh pathway has been reported to rely on two distinct mechanisms, namely canonical- and non-canonical- Hh activation (16). In the canonical pathway (figure 1 A), one of the Hh proteins binds to the hedgehog protein receptor Patched (Ptc), which is eventually internalized and degraded. Repression of the Ptc, occurring upon Hh binding, triggers 7-transmembrane protein Smoothened (Smo) activity, in turn promoting, downstream, GLI family zinc finger (Gli) nuclear traslocation. As a result, Gli modulates a plethora of genes widely identified as Hh targets (17), involved in cell cycle regulation, apoptosis, proliferation, angiogenesis, self-renewal, and epithelial-to-mesenchymal transition (22).

Besides, Gli are also regulated by a family of tumor suppressor proteins, namely Suppressor of Fused (SUFU) (23). When Ptc ligands are missing, Gli proteins are recruited by Sufu, which are in charge of inhibiting their nuclear translocation (24). For this reason, the full-length Gli proteins are converted to a C-terminal shorten repressed form (Gli-R). This structure is phosphorylated by glycogen synthase kinase 3 beta (GSK3β), casein kinase I (CK1), and
protein kinase A (PKA) (25). Gli proteins retained at the cytoplasm by Sufu are then degraded or processed, overall triggering Hh inhibition. However, how these last steps are operated in mammals are elusive still (23).

Non-canonical Hh activation (figure 1 B), on the other hand, has been characterized to be orchestrated by two separate pathways. Type I non-canonical Hh activation relies on Ptch1-activity when Ptc ligands are missing. This noncanonical signaling activity regulates the cell cycle through modulation of the subcellular localization of cyclin B1 (26). Type II non-canonical Hh activation is Smo-dependent Gli-independent. Small GTPases RhoA and Rac1 are the main players in charge for triggering this pathway, in a cellular-context manner (27-29). In carcinogenic processes these mechanisms have been reported to be profoundly affected as a result of Hh signaling misregulation (30). Given the role of Hh pathways in cell development, its aberrant activation might thus contribute to hematological malignancies progression, overall representing a promising strategy to target for developing novel drug-based approaches (31).

HEDGEHOG SIGNALING IN MULTIPLE MYELOMA

MM cells-mesenchymal stromal cells (MSCs) interactions have been described to play an outstanding role in MM pathogenesis, eventually contributing to MM cell survival, proliferation and chemoresistance (32). Shh produced by the stromal cells supports proliferation of hematopoietic stem cells, prompts germinal-center B cells survival and antibody production (33-35). In tumor context, MSC-induced Shh signaling is important in protecting my-

---

**Figures 1.** A. Canonical activation of Shh pathway. Canonical pathway is triggered by interaction between Shh and Ptch1. In response to this binding, Ptch1 no longer inhibits Smo, which in turn promotes downstream Gli nuclear translocation and target genes activation; B. Non-canonical Shh pathway. Non-canonical activation can be orchestrated by two separate pathways. Type I Smo-independent activation relies on Ptch interaction with cyclin B1, leading to cell cycle regulation. Type II is Smo-dependent Gli-independent. When Shh binds Ptch1, Smo activates Gi protein and small GTPases RhoA and Rac1, as well as calcium release stimulation from endoplasmic reticulum and PLC-γ-catalyzed the opening of IP3-depedent channels by the generation of IP3.
elodysplastic syndrome (MDS) cells from apoptosis (36) Despite accumulation of a plethora of genetic lesions, myeloma PCs lose their dependency on BM microenvironment only in the latest stages of disease and therefore long-term culture of primary MM cells without stromal support is rarely possible in vitro (37). Among the main MSC-released soluble factors contributing to myeloma cells survival, Shh allow survival and growth of MM cells. Indeed, its proliferative effect is inhibited by cyclopamine, an alkaloid which binds to SMO stabilizing its inactive conformation (37).

CD138+ cells from MM patients exhibit overexpression of Hh signaling components, such as PTCH, GLI1 and GLI2 through the activation of non-canonical Smo-independent pathway (16). Moreover, a significant down-regulation of Hh repressor gene GLI3 has been described in malignant plasma cells compared to the healthy counterpart (16). MM is characterized by two distinct populations: CD138-CD19+ stem cells, resembling memory B cells, and malignant CD138+CD19- terminally differentiated plasma cells (38). Peacock et. al (32) demonstrated a marked down-regulation of PTCH1 in CD138-CD19+ stem cell compartment, together with an increase of SMO and GLI1 expression. On the other hand, CD138+CD19- differentiated plasma cells showed increased PTCH1 levels. Therefore, CD138-CD19+ stem cell populations are more sensitive to Hh ligand than malignant CD138+CD19-terminally differentiated plasma cells (32).

In addition to the stromally induced Hh signaling, MM cells are able to produce and secrete themselves the Hh ligands. Autocrine Shh signaling enhances tumor proliferation and protects CD138+ cells from spontaneous and stress-induced apoptosis increasing BCL-2 expression levels (39). This evidence correlates with an independent study reporting that an Hh-gene signature is able to cluster MM patients in two subgroups characterized by the opposite Hh pathway expression in mature PCs and their precursors. In particular, patients with Hh hyperactivation in MM cells, but not in their B cells, show higher genomic instability associated to shorter progression-free survival and overall survival (40).

Hh signaling is also associated with the nuclear transcription factor-kB (NF-kB) pathway in several tumors such as liver cancer, breast cancer, prostate cancer, pancreatic cancer, diffuse large B-cell lymphoma (DLBCL) (41-44). NF-kB is a heterodimeric complex consisting of a p50 (NF-kB1) and p65 (RelA) subunits, which form an inactive cytoplasmic ternary complex with the inhibitory protein IKBa. In response to an extracellular stimulus, IKBa may be degraded and NF-kB can translocate into the nucleus to activate the expression of genes involved in the immune and inflammatory responses, such as interleukin 2 receptor alpha chain gene, interleukin 6, granulocyte colony-stimulating factor, interferon-beta (IFN-b) (45). Interestingly, it has been reported that canonical pathway activation of Sonic Hedgehog is responsible for the enhancement of NF-KB activity in MM cells, preventing their apoptosis (46).

Tumor-derived Hh signaling can favor the production of receptor activator of nuclear factor-kB ligand (RANKL) in osteoblasts, stimulating osteoblastogenesis and increasing bone resorption (47). Hh signaling has also been found to stimulate MSCs differentiation on osteoblasts regulating expression of Runt-related transcription factor (RUNX2) and Osterix (OSX) expression (48). Activation of osteoblastogenesis is directly modulated by SMO and GLIs-induced signaling (48). Indeed, inhibition of Shh signaling by using cyclopamine strongly reduces osteoblastogenesis (49). Myeloma PCs acts as GLI1 suppressor on MSCs, thus, reducing the potential of MSCs to differentiate in osteoblasts (50).

**TARGETING HH PATHWAY**

Given the crucial role played by Hh pathway in MM progression, recent reports focused on developing new therapeutic strategies aiming to its inhibition. One of them targets the Smo receptor using cyclopamine, eventually resulting in the inhibition of Hh signaling (51). Since Hh signaling regulates NF-kB through both its classical pathway (ShH/PTCH1/SMO/GLI1) and non-classical pathway by SMO recruitment of TRAF6 to ubiquitination, the SMO inhibitor cyclopamine in combination with bortezomib enhance the proteasome inhibitor-induced cytotoxic effects (46) These results enforce the hypothesis describing a proteasome-Hh axis which may be targeted in the feature studies. Despite the promising results, cyclopamine showed teratogenic potential, toxicity and poor bioavailability, overall discouraging further application aiming to clinical outcomes (52). However, cyclopamine opened the path towards further drug development aiming to target Hh pathway for MM treatment.
Currently, a newer drug namely Vismodegib acting through Hh pathway inhibition has been approved by US Food and Drug Administration’s (US FDA) priority review program on January 30th, 2012 for the treatment of advanced basal-cell carcinoma (BCC) (53). Since Vismodegib was found to have an acceptable safety profile and antitumor activity in patients with BCC and medulloblastoma (54, 55), new clinical trials are being planned in other malignances, including MM (table I). However, patients undergoing Vismodegib treatment against BCC showed bone toxicities, with premature fusion of the epiphyses reported in pediatric patients (56). Moreover, cramps or dysgeusia over the course of the therapy appeared in several patients, requiring interruption of the standard therapy, eventually shifting to an intermittent Vismodegib schedule (57).

In parallel, Sonidegib (Odomzo™), a SMO receptor antagonist, has being developed by Novartis for the treatment of BCC (58). This drug was reported to hamper cell viability, neurosphere formation, and Gli transcriptional activity, triggering the apoptotic cascade by activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase in vitro (59). In a transgenic mouse model of islet cell neoplasms Sonidegib significantly reduced tumour volume by 95% compared with untreated littermates by inhibition of Hh signaling (59). Given the efficacy and tolerability of a topical formulation of Sonidegib in BCC patients, phase I/II investigation are underway on other malignances including medulloblastoma, small cell lung cancer, breast cancer, myelofibrosis, chronic myeloid leukaemia, and MM (table I) (60-64). As for Vismodegib, clinical trials displayed a set of typical side effects associated with Sonidegib administration. Muscle spasms, alopecia, dysgeusia, nausea, increased Creatin Kinase, fatigue, decreased weight, diarrhea, decreased appetite, myalgia, and vomiting were frequent in patients, eventually undergoing dose interruptions, reductions, or treatment discontinuation (65). For this reason, further studies are needed to evaluate the usage and dosage of both of these Hh inhibitor for clinical approaches.
Because the GLI proteins are the final effectors of Shh pathway, the development of a GLI-targeted approach might be useful to inhibit tumor growth and therapy resistance. Among GLI antagonists, there are GANT58 and GANT61 (GLI-ANTagonist) (66) GANT61 is more specific toward GLI proteins and effectively reduces GLI1 and GLI2 DNA-binding ability. Arsenic Trioxide (ATO) (a Food and Drug Administration (FDA)-approved drug with sub-micromolar potency against GLI1/267 (67) was shown to inhibit GLI1 directly inhibiting its transcriptional activity (68). MM cells treated with ATO also show inhibition of NF-kB, hampered adhesion to MSCs with consequent disruption of tumor growth and survival (69). A first phase II study of ATO in a MM cohort was designed to assess the response to therapy of patients with relapsed or resistant MM, previously treated with autologous stem cell (70). Eligible patients (n = 10) received a 2-hour daily infusion of ATO 0.15 mg/kg for 60 days. The treatment was supplemented for 30 days more in patients showing a response, defined as a reduction in myeloma paraprotein at days 30 and 60. Three out of ten patients who completed more than 30 days of ATO infusion were characterized by >50% reduction in serum paraprotein levels (n = 2), a more stable disease (n = 1). Furthermore, one out of ten progressed. Surprisingly, five patients belonging to the initial cohort, displayed stable disease (n = 2) and progressed (n = 3), already upon < 30 days treatment. Table 1 lists completed clinical trials, providing a strong basis for the use of ATO in MM patients. Interestingly, ATO found an important clinical path in counteracting relapsed or refractory acute promyelocytic leukemia. However, ATO usage has been discouraged as a consequence of its side effects on healthy tissues, eventually resulting in cardiotoxicity (71). Notably, QT prolongation, torsades de pointes and sudden cardiac death have been reported upon ATO administration. The main reason behind ATO-related cardiac toxicity is related to the large amount of ROS produced following ATO treatment, which in cardiac cells, as a consequence of the low amount of antioxidants, it is enhanced (71, 72).

### Table 1. Clinical trials. The table reports registered clinical trial on https://clinicaltrials.gov focused on Hh inhibitors for MM treatment.

<table>
<thead>
<tr>
<th>DRUG</th>
<th>TRIAL REGISTRATION NUMBER</th>
<th>LOCATION</th>
<th>YEARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATO (Arsenic Trioxide)</td>
<td>NCT00469209</td>
<td>U.T.M.D Anderson Cancer Center Huston, Texas, US</td>
<td>2006-2008</td>
</tr>
<tr>
<td></td>
<td>NCT00258245</td>
<td>Barbara Ann Karmanos Cancer Institute Detroit, Michigan, US</td>
<td>2005-2008</td>
</tr>
<tr>
<td></td>
<td>NCT00201695</td>
<td>Ohio State University Columbus, Ohio, US</td>
<td>2004-2008</td>
</tr>
<tr>
<td></td>
<td>NCT0006021</td>
<td>Mount Sinai Comprehensive Cancer Center at Mount Sinai Medical Center Miami Beach, Florida, US</td>
<td>2000-2007</td>
</tr>
<tr>
<td></td>
<td>NCT000193544</td>
<td>City of Hope Duarte, California, US</td>
<td>2005-2009</td>
</tr>
<tr>
<td>Vismodegib (GDC-0449)</td>
<td>NCT02465060</td>
<td>University of Alabama at Birmingham Cancer Center Birmingham, Alabama, US</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td>NCT03297606</td>
<td>Cross Cancer Institute Edmonton, Alberta, CA, US</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td>NCT03878524</td>
<td>OHSU Knight Cancer Institute Portland, Oregon, US</td>
<td>Recruiting</td>
</tr>
<tr>
<td></td>
<td>NCT02086552</td>
<td>Mayo Clinic Rochester, Minnesota, US</td>
<td>2014-2021</td>
</tr>
</tbody>
</table>

Further approaches to enhance Hh inhibitors efficiency include synergic strategies with molecules targeting the HH signaling cascade at multiple levels. With this regard such ATO has been recently tested together with Itraconazole, Vismodegib or Sonidegib (73).
For this reason, it should not be surprising if multiple combinations of Hh-targeting agents will be disclosed soon. For this purpose, we listed the literature currently available investigating the cross-talk between Hh and multiple myeloma in table II.

**CONCLUSION AND FUTURE PERSPECTIVES**

MM is a hematological disease characterized by an aberrant activation of several molecular mechanisms, eventually reshaping the bone microenvironment, and resulting in MM progression. In this landscape, researchers are aiming to identify novel therapeutic targets to improve patients’ prognosis. With this regards, Hh activation has been reported to cover an underestimated role in bone marrow development, thus prompting different groups to target this cascade in different hematological diseases (74). In the MM context, Hh aberrant signaling, in turn mediated by Shh release, affects bone marrow microenvironment transformation, supporting the proliferation of malignant plasma cells by enhancing NF-κB signaling, also resulting in chemotherapy resistance (75). In this context, targeting the Hh pathway may represent a valuable strategy. To date, the main strategies are represented by three drugs (ATO, Vismodegib, and Sonidegib) which are currently being tested in different clinical trials (table I). However, the usage of drugs targeting Hh cascade may not be useful enough to hamper MM progression. For this reason, a further effort could be done to design more powerful Hh modulators. In alternative, the ones currently available may be tested together with molecules targeting Hh cascade on different levels to enhance Hh modulators’ effect. Ultimately, an outstanding strategy may also be represented by supplementation of currently clinical-available drugs in combination with Hh modulators, aiming to obtain a more beneficial treatment. In this regard administration of Ixazomib reshapes the MM microenvironment also stimulating Hh cascade. However, further studies are needed to fully understand the regulatory mechanisms underlying Hh signaling pathway and how PIs affect them, towards the development of new treatment to efficiently hamper MM progression.

<table>
<thead>
<tr>
<th>REFERENCE</th>
<th>TITLE</th>
<th>JOURNAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>A novel Bruton’s Tyrosine Kinase inhibitor CC-292 in combination with the proteasome inhibitor carfizomib impacts the bone microenvironment in a multiple myeloma model with the resultant antmyeloma activity.</td>
<td>Leukemia, 2014</td>
</tr>
<tr>
<td>34</td>
<td>Sonic Hedgehog is produced by follicular dendritic and protects germinal center B cells from apoptosis.</td>
<td>Journal of Immunology, 2005</td>
</tr>
<tr>
<td>40</td>
<td>Opposite activation of the Hedgehog pathway in CD138+ plasma cells and CD138- 19+ B cells identifies two subgroups of patients with multiple myeloma and different prognosis.</td>
<td>Leukemia, 2016</td>
</tr>
<tr>
<td>46</td>
<td>Targeting the cross-talk between the Hedgehog and NF-kappaB signaling pathways in multiple myeloma.</td>
<td>Leukemia &amp; Lymphoma, 2019</td>
</tr>
<tr>
<td>47</td>
<td>The role of Hedgehog signaling in tumor induced bone disease.</td>
<td>Cancers (Basel), 2015</td>
</tr>
<tr>
<td>50</td>
<td>Ixazomib improves bone remodeling and counteracts Sonic Hedgehog signaling inhibition mediated by myeloma cells.</td>
<td>Cancers (Basel), 2020</td>
</tr>
<tr>
<td>75</td>
<td>Effect of Hedgehog pathway abnormality on chemotherapeutic resistance of multiple myeloma.</td>
<td>Zhongguo Shi Yan Xue Ye Xue Za Zhi, 2017</td>
</tr>
</tbody>
</table>

Table II. Currently available literature investigating Hh-MM crosstalk. The table reports the available works investigating the role played by Hh in MM progression as they are cited along the main body of the text.
ACKNOWLEDGMENTS

This study was supported in part by AIL. (Associazione Italiana contro le Leucemie) sezione di Catania, FON.CA.NE.SA. (Fondazione Catanese per lo Studio delle Malattie Neoplastiche del Sangue).

ETHICS

Conflicts of interests
The authors have declared no conflict of interests.

Fundings
This study was supported by Piano di Incentivi per la ricerca di Ateneo 2020/2022 Linea di intervento 2 (FDR). This study was supported by Piano di Incentivi per la ricerca di Ateneo 2020/2022 Linea di intervento 3 Starting grant (DT). CG was supported by the PON AIM R&I 2014-2020-E68D19001340001. N.V. was supported by the PON AIM R&I 2014-2020-E66C18001240007.

Authors’ contribution
Conceptualization: ID, DT, CG, AR, SG, NV, FDR, GAP; validation: AR, GS, AB, LL, ID; writing-original draft preparation: ID, DT, CG, SG, GAP, ELS, NV, TZ, FDR, GLV, RP, RP; supervision: DT, GAP, DLF, FDR, NV, DC, RP, GLV, RA, ID, GLV. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials
No new data were generated or analysed in this research.

Ethical approval
N/A.

REFERENCES


