



Universiteit  
Leiden  
The Netherlands

## **TGF- $\beta$ family signaling in endothelial cells and angiogenesis**

Ma, J.

### **Citation**

Ma, J. (2021, September 30). *TGF- $\beta$  family signaling in endothelial cells and angiogenesis*. Retrieved from <https://hdl.handle.net/1887/3214214>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3214214>

**Note:** To cite this publication please use the final published version (if applicable).

# **Chapter 7**

**General discussion**

Endothelial cells (ECs) constitute the inner layer of arteries, veins and capillaries and their response to the biological, chemical and mechanical cues in blood and microenvironment strongly determines one's health condition. The TGF- $\beta$  family proteins are among the many cytokines that regulate EC behaviour. The activation of TGF- $\beta$  signaling can trigger the conversion of ECs from a cobblestone morphology into a mesenchymal-like cell type with fibroblast appearance. This process is termed endothelial-to-mesenchymal transition (EndMT). During embryonic stages, EndMT is pivotal for the development of cardiovascular system and alteration of embryonic EndMT often leads to prenatal lethality. Postnatally, EndMT has been causally linked to the occurrence and development of pathological processes in multiple diseases. In addition, activation of the EndMT process can potentially be applied in tissue engineering. New insights into the mechanisms by which TGF- $\beta$  family members control EndMT may provide therapeutic opportunities. In this thesis, we elucidated the role of the transcription factors SNAIL and inhibitor of DNA-binding proteins (ID1) in TGF- $\beta$ /BMP-induced EndMT. We also describe in detail the methodology to investigate the role of TGF- $\beta$  family members in the functional regulation of ECs and breast cancer cell types. What's more, we synthesized and identified two novel macrocyclic BMPRI inhibitors and assessed their inhibitory effects on ECs function, including homeostatic angiogenesis using zebrafish embryos. Finally, we also examined their ability to inhibit breast cancer cells induced angiogenesis using a zebrafish xenograft breast tumor model.

### **TGF- $\beta$ -induced EndMT and its role in disease and tissue Engineering**

In **Chapter 2** we provided with an overview of TGF- $\beta$ -induced EndMT in disease and tissue engineering. We summarized the mechanisms of the TGF- $\beta$  signaling pathway in ECs. TGF- $\beta$ , a potent inducer of EndMT, activates SMAD and non-SMAD signaling pathways in ECs. TGF- $\beta$  signaling also interacts with other signaling cascades, which may enforce, modulate or inhibit the ability of TGF- $\beta$  to promote EndMT. EndMT has been linked to fibrosis, cancer and cerebral cavernous malformations (CCM) as detailed in **Chapter 2**, while the number of diseases and biological processes to which EndMT has been associated is only increasing. For example, a recent study showed that the occurrence of EndMT promotes the pathological development of subretinal fibrosis, the end-stage of age-related macular degeneration (AMD) that results into permanent vision loss [1]. TGF- $\beta$  drives both EMT and EndMT in the eye [2, 3]. Therefore, the identification of the molecular mechanisms underlying TGF- $\beta$ -induced EndMT may contribute to the development of therapies for AMD. In addition, the precise monitoring of EndMT and its reversibility will contribute to gain insights into the application of EndMT for tissue engineering. Concerning this latter aspect, it is of interest to note that TGF- $\beta$ -induced EndMT has been suggested to promote angiogenesis. Li et al. demonstrated that the stimulation of EndMT in HUVECs induces angiogenesis by upregulating VEGF secretion [4]. Thus, TGF- $\beta$  regulated EndMT could also be used to induce vessel ingrowth in angiogenesis-related pathological conditions. In summary, EndMT or its reverse process mesenchymal-to-endothelial transition (MET), could be pharmacologically targeted or exploited for therapeutic and tissue regeneration purposes.

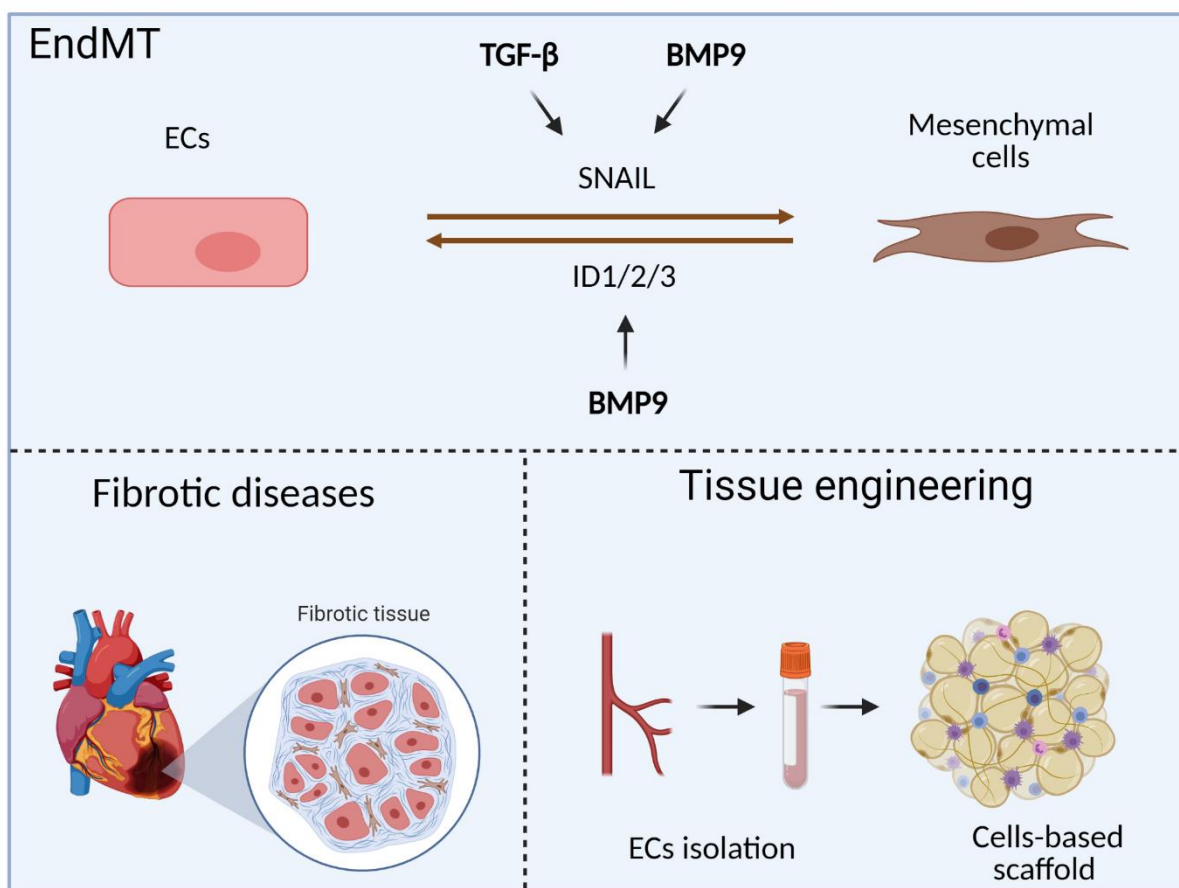
### **Methods to investigate TGF- $\beta$ -induced EndMT and its mediators using CRISPR/CAS9 gene editing**

In **Chapter 3** we described methods to investigate the underlying molecular mechanisms by which cytokines, such as TGF- $\beta$ , induce or regulate EndMT. A detailed protocol was provided on how to stimulate and monitor the occurrence of EndMT. The striking changes in cell morphology and the expression of EndMT-related markers after TGF- $\beta$  treatment are considered hallmarks for EndMT. We provided with an immunofluorescent staining protocol to assess the change in the expression of EndMT markers. This assessment can be dynamically visualized using a fluorescence-based live-cell imaging approach. For example, the Lifeact-GFP, which labels the filamentous actin (F-actin) structures in living cells to monitor its dynamic changes, can be applied to observe the process of EndMT/EMT [5, 6]. Another way to monitor dynamic changes in endothelial and mesenchymal marker expression in real time is to engineer ECs to express fluorescent markers into the endogenous locus of endothelial and mesenchymal markers using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CAS9) mediated gene editing. This strategy can not only be used to obtain mechanistic insights, but also can be used to screen and identify potential drugs that have promoting or inhibitory effects on EndMT [7]. Of note, by assessing the influence on the expression of the EndMT markers F-actin and  $\alpha$ -SMA in HUVECs, Song *et al* identified the compound CHIR-99021 with potential to inhibit radiation-induced pulmonary fibrosis (RIPF). CHIR-99021 can be used to antagonize radiation-induced EndMT at tumor sites, thereby enhancing the therapeutic effect of ionizing radiation (IR) on non-small cell lung cancers [8]. Besides describing how to study the effects of cellular factors on EndMT, we also disclosed how downstream effectors or modulators of EndMT can be studied using the method of CRISPR/CAS9-mediated gene editing. Functional inactivation of SNAIL, which is indispensable for TGF- $\beta$ -induced EndMT, was used as a proof of principle. The introduced a workflow that can be applied to other putative EndMT mediating/modulating factors to confirm their functional involvement in this process. Furthermore, to identify novel factors that are involved in regulating TGF- $\beta$ -induced EndMT, cell lines that express a fluorescent reporter under control of (preferably endogenous) promoter of a gene encoding an endothelial and/or mesenchymal marker can be used in combination with a genome wide guide screens with CRISPR/CAS9 knockout libraries and fluorescence activated cell sorting (FACS). Genes that become enriched or depleted upon TGF- $\beta$ -induced EMT might be possible modulators of the EndMT process.

## Unravelling the mechanisms of SNAIL and ID determining TGF- $\beta$ -induced EndMT

How different TGF- $\beta$  family proteins functionally interact to fine tune EndMT is still unclear. To gain deeper insights into this process, we compared in **Chapter 4** the effects of TGF- $\beta$ 2 and BMP9 on EndMT. We used murine endothelial MS-1 and 2H11 cells to find that both TGF- $\beta$ 2 and BMP9 stimulate SNAIL and SLUG expression, while only TGF- $\beta$ 2 induces EndMT. We reasoned that BMP9 fails to trigger EndMT as it potently induces the expression of genes that encode for ID proteins in ECs (Figure 1). Previous reports have suggested controversial functions of ID proteins in EMT and their role in EndMT remained unknown. High ID1 expression has been linked with more EMT and/in? advanced cancer stages in bladder and lung cancer patients [9, 10]. On the contrary, ID proteins were found to prevent EMT induction by dimerizing with the transcription factor E2A to antagonize the E2A-mediated inhibition of epithelial proteins expression [11]. Moreover, the negative function of ID proteins in EMT was

also observed in NMuMG mammary epithelial cells [12]. Using two different in vitro EC line models, our study revealed that ID1/2/3 proteins are essential for maintaining endothelial properties and to antagonize SNAIL, thereby preventing EndMT. To determine the general significance of this observation, additional EC types need to be analyzed. Furthermore, it will be of interest to extend these studies by investigating the SNAIL/ID1 interplay in physiological or pathological models of EndMT or in clinical patient samples in the future. The understanding of how different factors control and fine-tune EndMT in temporal- and spatial- dependent manner in normal (embryonic) tissues and pathological processes may help to manipulate this process for therapeutic gain in fibrosis and cancer therapy, and even facilitate tissue engineering applications.



**Figure 1.** The effects of TGF- $\beta$  and BMP9 on EndMT. TGF- $\beta$  induces EndMT by promoting SNAIL expression. BMP9 promotes SNAIL and ID1/2/3 expression and is unable to stimulates EndMT. The precise control of EndMT may be used to treat fibrotic diseases and in tissue engineering.

### Establishing Zebrafish xenograft models to investigate cancer progression

In **Chapter 5**, we summarized different xenograft models in embryonic zebrafish to investigate the mechanisms by which signaling initiated by TGF- $\beta$  family members control breast cancer cell intravasation, extravasation and how they regulate tumor angiogenesis. The xenograft zebrafish embryo system shows obvious advantages, such as (1) efficient, low-cost and time-saving in vivo model; (2) many key signaling processes in humans are conserved in zebrafish, such as TGF- $\beta$  and BMP signaling [13, 14]; (3) transgenic (deficient in pigment) zebrafish lines and fluorescently labelled cancer cells can be used to visualize ECs/blood vessels and

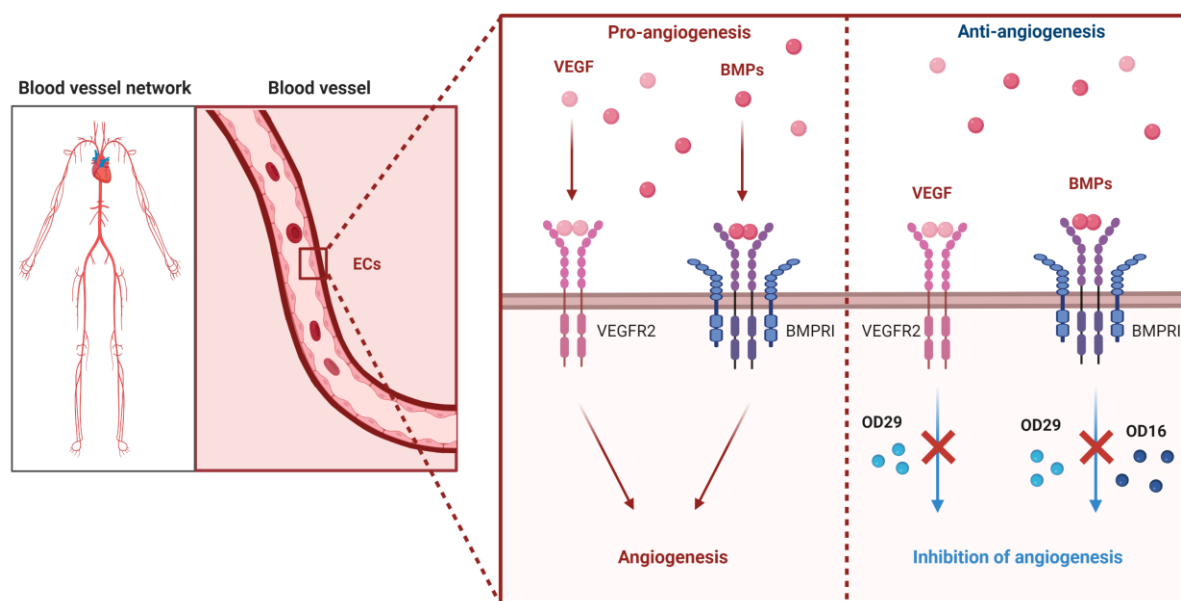
cancer cell behaviour; (4) human and murine cells are well tolerated by the immature immune system in zebrafish embryos; (5) it can be used in high throughput screens with pharmacologically active compounds, as small molecules can penetrate into embryos easily. However, disadvantages of the xenograft zebrafish model also need to be taken into consideration. For example, injected zebrafish are incubated at a temperature that raises from 28°C to 33°C-35°C. This may cause metabolic changes in both zebrafish host and injected human cancer cells (normally cultured at 37 °C).

Personalized assessment of the differential sensitivity and resistance of tumor cells to therapeutic drugs can greatly help to improve the treatment options, resulting in a better quality of life for cancer patients. Importantly, patient-derived xenograft (PDX) zebrafish models have been used in pre-clinical setting to predict individual patient responses to chemotherapy and radiotherapy [15, 16]. The tested cancer cell chemosensitivity profiles obtained in zebrafish and mouse xenografts were found to be highly consistent with each other. Whereas the zebrafish PDX predicted 80% correctly the patient clinical outcome [17], the PDX mouse model predicted 87% correctly the patient drug response in the clinic [18]. Comparing with the existing patient-derived *Avatars*, the patient-derived Avatars created by xenografting into zebrafish embryos takes less time (a week) than organoids (4-5 weeks) and mice models (months). The faster predication can help patients to avoid unnecessary trials and improve therapeutic efficiency. Furthermore, to assess tumor growth the zebrafish xenograft needs fewer patient tumor cells (300-500 cells) than the mouse xenograft ( $1 \times 10^6$  cells). Statistical analysis is also easier in zebrafish models, as reaching enough power becomes more feasible, especially when tumor biopsies are limiting in cell volume. Until now, patient-derived breast cancer and pancreatic cancer models have already successfully been developed and tested in zebrafish. We anticipate that this will ultimately contribute to guide clinical personal treatments [19, 20].

## Two novel BMPRI inhibitors that inhibit ECs function

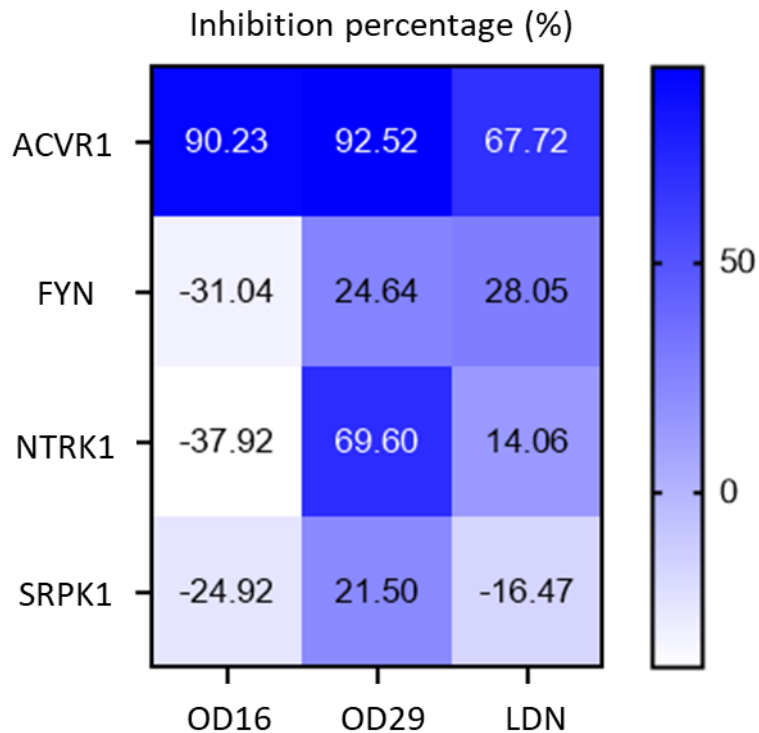
In order to develop new potential anti-angiogenesis drugs, we synthesized and identified two macrocyclic compounds, which we termed OD16 and OD29 (described in **Chapter 6**). Both OD16 and OD29 exhibited inhibitory signaling activity against BMPRI, specifically ALK1/2. In addition, we also found that OD29 inhibited VEGF-induced extracellular regulated kinase (ERK) MAP kinase phosphorylation (see also discussion further below) (Figure 2). Treatment studies with the compounds revealed that both compounds impaired EC angiogenesis *in vitro* and decreased homeostatic and tumor-induced angiogenesis *in vivo*. Interestingly, previous research reported controversial roles of ALK1 and ALK2 on angiogenesis. On the one hand, ALK1/2 were described to mediate angiogenesis as an ALK1 neutralizing antibody reduced ECs sprouting and the endothelial specific ALK2 knockdown blocked retinal angiogenesis in mice [21, 22]. On the other hand, both ALK1 and ALK2 were also found to inhibit angiogenesis. For example, the activation of ALK1 signaling prevented retinal vascularization, while the knockdown of ALK2 in HUVECs favoured their sprouting [23, 24]. As OD16 and OD29 efficiently antagonized angiogenesis in zebrafish, our results suggest that ALK1/2 kinase activity is needed for angiogenesis. These two compounds may help to interrogate the functional roles of the kinases that they target in vascular or other biological processes. Importantly, the off-targeting of small molecules is often an issue that needs to be taken into consideration. The results obtained with compounds should be complemented with specific

genetic knockdown of ALK1 and/or ALK2 to provide additional insight into their role in angiogenesis.



**Figure 2.** The BMPRI inhibitor OD16 and the dual BMP and VEGF signalling inhibitor OD29 prevent VEGF or/and BMPs stimulated angiogenesis.

As aforementioned, we also found that OD29 inhibits VEGF-induced ERK MAP kinase phosphorylation without influencing phospho-VEGFR in ECs. To unveil the potential target of OD29 upstream of phosphorylated ERK, we analyzed the *in vitro* kinase inhibition profiles of these two compounds. Comparing with OD16 and the well characterized BMP type I receptor kinase inhibitor LDN-193189, OD29 demonstrated a strong inhibitory potential in targeting serine-arginine protein kinase 1 (SRPK1), proto-oncogene tyrosine-protein kinase FYN (FYN) and neurotrophic receptor tyrosine Kinase 1 (NTRK1), which might be the reason for its inhibitory activity on VEGF-induced ERK phosphorylation (Figure 3). SRPK1 is a cellular kinase that regulates gene splicing and is an upstream activator of ERK. It has been shown that the over-expression of SRPK1 increases the phosphorylation of ERK in gastric cancer cells [25]. The knockdown of SRPK1 reduces VEGF expression in melanoma cells [26]. SRPK1 is highly expressed in ECs (as analyzed by human protein atlas), but so far it has not been implicated as a downstream kinase activated in response to VEGF in ECs [27]. Inhibition of the non-receptor tyrosine kinase FYN led to a decrease in ERK phosphorylation in mouse aortic endothelial cells (MAECs) and in imatinib resistant chronic myelogenous leukemia (IM-R) cells [28, 29]. Moreover, Somwar *et al.* showed that the inhibitors of type I NTRK (larotrectinib and altiratinib) suppress the NTRK1 phosphorylation and also inhibit ERK1/2 phosphorylation in Ba/F3 cells [30]. The NTRK1 is expressed in ECs at the eye and heart [27]. Also NTRK1 action as downstream kinase in response to VEGFR signaling has not been demonstrated. These targets as well as additional potential OD29 targets should be explored to explain its inhibitory effect on VEGF-induced pERK.



**Figure 3.** The inhibition percentage (%) of OD16, OD19 and LDN-193189 at 0.1 $\mu$ M on kinase activity, including ACVR1, FYN, NTRK1 and SRPK1.

The anti-angiogenic effects of OD16 and OD29 in transgenic Casper zebrafish (fli:EGFP) during embryonic development and tumor cell-induced blood vessel formation emphasize their potential for clinical application. The anti-angiogenesis effect of these two inhibitors could be further validated in mouse cancer models, for example, the RIP-Tag2 transgenic mouse, in which the mouse generates insulinomas that are highly dependent on angiogenesis for rapid tumor growth [31, 32].



## Reference

1. Shu, D.Y., E. Butcher, and M. Saint-Geniez, *EMT and EndMT: emerging roles in age-related macular degeneration*. International journal of molecular sciences, 2020. **21**(12): p. 4271.
2. Piera-Velazquez, S. and S.A. Jimenez, *Endothelial to mesenchymal transition: role in physiology and in the pathogenesis of human diseases*. Physiological reviews, 2019. **99**(2): p. 1281-1324.
3. Tamiya, S., L. Liu, and H.J. Kaplan, *Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact*. Investigative ophthalmology & visual science, 2010. **51**(5): p. 2755-2763.
4. Cao, S.-J., L. Hong, and X.-Q. Li, *Mechanistic studies on the role of TGF- $\beta$ 1 in angiogenesis through EndMT*. Vascular, 2020: p. 1708538120953668.
5. Riedl, J., et al., *Lifeact: a versatile marker to visualize F-actin*. Nature methods, 2008. **5**(7): p. 605-607.
6. Malinverno, M., et al., *Endothelial cell clonal expansion in the development of cerebral cavernous malformations*. Nature communications, 2019. **10**(1): p. 1-16.
7. Richardson, F., et al., *The evaluation of E-Cadherin and vimentin as biomarkers of clinical outcomes among patients with non-small cell lung cancer treated with erlotinib as second-or third-line therapy*. Anticancer research, 2012. **32**(2): p. 537-552.
8. Song, Y., et al., *Identification of radiation-induced EndMT inhibitors through cell-based phenomic screening*. FEBS open bio, 2019. **9**(1): p. 82-91.
9. Hu, H., et al. *A novel role of Id-1 in regulation of epithelial-to-mesenchymal transition in bladder cancer*. in *Urologic Oncology: Seminars and Original Investigations*. 2013. Elsevier.
10. Castañón, E., et al., *The inhibitor of differentiation-1 (Id1) enables lung cancer liver colonization through activation of an EMT program in tumor cells and establishment of the pre-metastatic niche*. Cancer letters, 2017. **402**: p. 43-51.
11. Massari, M.E. and C. Murre, *Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms*. Molecular and cellular biology, 2000. **20**(2): p. 429-440.
12. Kondo, M., et al., *A role for Id in the regulation of TGF- $\beta$ -induced epithelial-mesenchymal transdifferentiation*. Cell Death & Differentiation, 2004. **11**(10): p. 1092-1101.
13. Kohli, G., et al., *Cloning of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and its type II receptor from zebrafish ovary and role of TGF- $\beta$ 1 in oocyte maturation*. Endocrinology, 2003. **144**(5): p. 1931-1941.
14. Nikaido, M., et al., *Conservation of BMP signaling in zebrafish mesoderm patterning*. Mechanisms of development, 1997. **61**(1-2): p. 75-88.
15. Mercatali, L., et al., *Development of a patient-derived xenograft (PDX) of breast cancer bone metastasis in a zebrafish model*. International journal of molecular sciences, 2016. **17**(8): p. 1375.
16. Costa, B., et al., *Zebrafish avatars towards personalized medicine—A comparative review between avatar models*. Cells, 2020. **9**(2): p. 293.
17. Fior, R., et al., *Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts*. Proceedings of the National Academy of Sciences, 2017. **114**(39): p. E8234-E8243.
18. Izumchenko, E., et al., *Patient-derived xenografts effectively capture responses to oncology therapy in a heterogeneous cohort of patients with solid tumors*. Annals of Oncology, 2017. **28**(10): p. 2595-2605.
19. Corsinovi, D., et al., *Zebrafish Avatar to Develop Precision Breast Cancer Therapies*. Anti-cancer Agents in Medicinal Chemistry, 2021.

20. Wang, L., et al., *Patient-derived Heterogeneous Xenograft Model of Pancreatic Cancer Using Zebrafish Larvae as Hosts for Comparative Drug Assessment*. Journal of visualized experiments: JoVE, 2019(146).
21. Seki, T., J. Yun, and S.P. Oh, *Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling*. Circulation research, 2003. **93**(7): p. 682-689.
22. Lee, H.-W., et al., *Alk2/ACVR1 and Alk3/BMPRI1 provide essential function for bone morphogenetic protein-induced retinal angiogenesis*. Arteriosclerosis, thrombosis, and vascular biology, 2017. **37**(4): p. 657-663.
23. Benn, A., et al., *Role of bone morphogenetic proteins in sprouting angiogenesis: differential BMP receptor-dependent signaling pathways balance stalk vs. tip cell competence*. The FASEB Journal, 2017. **31**(11): p. 4720-4733.
24. Larrivé, B., et al., *ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway*. Developmental cell, 2012. **22**(3): p. 489-500.
25. Xu, X., et al., *Serine-arginine protein kinase 1 (SRPK1) is elevated in gastric cancer and plays oncogenic functions*. Oncotarget, 2017. **8**(37): p. 61944.
26. Gammons, M., et al., *Targeting SRPK1 to control VEGF-mediated tumour angiogenesis in metastatic melanoma*. British journal of cancer, 2014. **111**(3): p. 477-485.
27. Uhlén, M., et al., *Tissue-based map of the human proteome*. Science, 2015. **347**(6220).
28. Fenouille, N., et al., *Persistent activation of the Fyn/ERK kinase signaling axis mediates imatinib resistance in chronic myelogenous leukemia cells through upregulation of intracellular SPARC*. Cancer research, 2010. **70**(23): p. 9659-9670.
29. Choi, S., et al., *KCa3. 1 upregulation preserves endothelium-dependent vasorelaxation during aging and oxidative stress*. Aging cell, 2016. **15**(5): p. 801-810.
30. Somwar, R., et al., *NTRK kinase domain mutations in cancer variably impact sensitivity to type I and type II inhibitors*. Communications biology, 2020. **3**(1): p. 1-13.
31. Cunha, S.I., et al., *Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis*. Journal of Experimental Medicine, 2010. **207**(1): p. 85-100.
32. Hu-Lowe, D.D., et al., *Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies*. Cancer research, 2011. **71**(4): p. 1362-1373.

