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CRISPR/Cas9 approach for creating a simplified cellular model to study the Desmoid cells in response to external stimuli and in cellular communication.

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Desmoid-type fibromatosis (DF) is a rare myofibroblastic benign tumor, that occurs sporadically or associated with familial adenomatous polyposis (FAP). Mutations in the beta-catenin (CTNNB1) gene are found in approximately 85% of sporadic desmoid cases; mutations in adenomatous polyposis coli gene (APC) have been linked to the genetic disorder, familial adenomatous polyposis, in which at least 25% of patients develop desmoid tumors. Both of these genes are involved in the Wnt signaling pathway.

β-catenin is a component of the cell adhesion junctions and the key mediator of the Wnt pathway, tightly regulated through a cytoplasmic multiprotein complex. Many types of alterations in the Wnt pathway as well as microenvironmental factors can cause an increase of β-catenin levels and its accumulation into the nucleus, activating the signaling cascade. Nuclear beta-catenin is the diagnostic marker of DF; we demonstrated that □-catenin is not the sole component of the complex accumulating in the nucleus of DF cells, but also the enzyme GSK-3□□is exclusively nuclear and is associated with □-catenin, and its nuclear localization is independent of □-catenin phosphorylation.

Desmoid tumors are characterized by a heterogeneous and variable clinical course with cases of continuous growth, fluctuation, stabilization and regression of the tumor. This clinical heterogenous outcome of the desmoid tumor is analogous to what we observed throughout our experiments in which primary culture of different DF cell samples showing a shared response to external factors but with a considerable degree of variability. In particular we demonstrated that the inflammatory cytokine, TGF-beta, through the canonical Smad-pathway, promotes growth of DF cells in vitro and stimulates the myofibroblast differentiation and the development of contractile stress fibers.

Moreover, primary cultures from desmoid tumor biopsies are characterized by concomitant presence of DF neoplastic cells and normal activated fibroblasts leading difficult to isolate DF cells alone and select their peculiarities.

For these reasons we planned to create a more controlled environment where to study the effects of isolated genetic changes in the Wnt pathway and the effect that these changes have on the response to external stimuli. More in particular, we decided to use the CRISPR/Cas9 system to create desmoid-like cell models that ensure genomic and phenotypic stability and that could mimic DF cells behavior. We planned to develop stable cell lines with mutations in the APC and CTNNB1 genes. For this purpose, we used BJ and Detroit cell lines and the plasmid eSpCas9-2A-Puro (PX459) V2.0 system in order to create CRISPR-Cas9 sgDNAs targeting the APC knock-out and the CTNNB1 knock-in introducing single base mutation in two of the major phosphorylation sites to induce T41A and S45F aminoacidic changes.

After single cell clonal isolation of APC knock-out, we selected APC^{KO} BJ cell clones and immunofluorescence analyses showed that the average proportion of APC-positive cells was 63%, meaning that 37% of selected cells are APC^{KO}. Moreover, as previously observed in desmoid cells, most of the APC^{KO} BJ cells and few CTNNB1 knock-in BJ cells showed a prevalent nuclear beta-catenin staining, largely colocalizing with GSK-3.

Previously we demonstrated that TGF-beta play a key role in proliferation and myofibroblast differentiation of desmoid cells organized in a fibrotic structure with the involvement of cell-cell and cell-matrix complex and showing colocalization of phospho-Fak with alpha-SMA in the focal contact sites. APC^{KO} BJ cells showed cytoplasmic localization of alpha-SMA protein and thin fibrillary structure, while phospho-FAK is expressed in the cell cytoplasm with strong staining in focal adhesion; after TGF-beta treatment p-Fak showed cytosolic expression with several focal contact colocalizing with alpha-SMA.

We are focusing our studies on the cellular communications with ECM, the cell adhesion receptors, and the role of focal-adhesion formation in TGF-beta stress-fiber assembly and myofibroblast differentiation. Since integrins link the extracellular matrix with the cytoskeleton creating a complex network and focal adhesion assembles, we are studying the involvement of TGF-beta in focal adhesion formation.

Integrins bind extracellularly to the ECM and intracellularly to the cytoskeleton, providing a dynamic interface between the cell and ECM by regulating inside-out and outside-in signaling. TGF-beta in its latent form, can be activated by its binding to integrins, otherwise it can in turn affect cell adhesion by regulating the expression of integrins and their ligands.

Preliminary data showed that alpha5 and alpha2 integrin subunits localized specifically in focal contact site in DF cells, while alpha5 and beta5 integrin subunits showed widespread surface localization, independently from the treatment. Interestingly alpha11 integrin appeared not expressed in wild-type cells, while it was well expressed in untreated and TGF-beta treated DF cells.

Our aim is to study the created cell lines, their behavior and their response to external stimuli, by investigating the gene and protein expression pattern. In particular we are studying the role of the TGF-beta, cell-cell contact and the receptors in the APC knock-out and CTNNB1 knock-in cells.