A novel Granule Cell Progenitors culture method as a new tool for investigating Hedgehog and other pathways relevant to cerebellar development and tumorigenesis.

SUMMARY

The Hedgehog (Hh) signaling is an essential pathway involved in development and cancer. Via the interaction with the 12-span transmembrane receptor Ptch, Hh ligands (Sonic, Desert and Indian Hh) release the activity of the 7-span membrane protein Smo. Yet uncompletely characterized molecular mechanisms, involving postranslational modifications of Smo and its shuttling within the primary cilium, translate the signal into the activation of the Gli family of transcription factors. The interplay between the three Gli members (Gil1, 2 and 3) regulates Hh-dependent transcription of a number of gene targets, many of which are master regulators of cell proliferation, such as N-Myc and Cyclin-D.

Hh pathway plays a pivotal role in cerebellar postnatal development, where it appears the main promoter of granule cell progenitors (GCPs) expansion, which in the mouse occurs between postnatal day (P)1 and P14, the time frame in which Hh signalling in the cerebellum raises up to its maximum (P7) and declines. By P14-P21 cerebellar maturation is completed. Silencing of Hh activity at the proper developmental time is molecularly regulated at multiple levels, some of which are still partially obscure, and appears to be essential to prevent transformation of GCPs into medulloblastoma.

Despite intense investigations, several aspects of Hh pathway activation and deactivation are not completely understood or conflicting interpretations exist. This is at least partially due to lack of appropriate models for the study of this pathway, *in vitro*. Indeed, Hh pathway gets quickly repressed in cell cultures, both in normal or cancer cells. Only transient cultures of freshly explanted primary granule cells (GC) allows Hh pathway investigation in a “naive” context, but only for few days.

Since this has long represented a big limitation in the field, we have recently developed a new procedure for GCPs colture, which overcome the limits of currently available models (transients cell cultures among other problems). Under the hypothesis that eliminating Hh-inhibiting growth factors and under the direct stimulation of Hh signal in the cell colture medium, GCs could be grown as neurosphere, we indeed generated a new primary model of GCPs with high and continuosly active Hh signalling.

Under defined concentrations of the SMO agonist SAG, cells explanted from P7 cerebelli can be undefinetely grown in colture as neurospheres. These neurospheres demonstrate complete activation overtime of the main effectors of Hh pathway Gli1 and N-Myc. They further show self-renewal capability *in vitro*, and constitutive expression of stemness genes, such as POU3f2, POU5f1, NANOG, and SOX2. These cultures do not get transformed during long periods in culture, as suggested by their continuous dependence on SAG.

SAG-dependent neurospheres express ZIC1, ATOH1 and NESTIN, which define their origin as GCPs. Consistently, these cells may be induced to differentiate into a homogeneous population of mature GC, expressing specifics markers such as TUBB3 and GABRA 6. Moreover, SAG-dependent cultures cannot be generated by the subventricular zone, but only from the mouse cerebellar explants taken at P1-P7.

Primary and continuously growing neurospheres of the same type can be generated by GC explanted from Ptch KO animal, even without SAG, further suggesting that, in the absence of specific Hh inhibitory factors, constitutive Hh activation is the only element required for their growth and survival.

Overall, these data suggest that using culture conditions that mimic the appropriate biological signalling, allowed the isolation a of transient amplifying GCP population in vitro, which is able to propagate in culture for undefined periods of time. This model represents a novel and important tool to study issues related to physiological and pathological aspects of Hh-dependent GCPs growth and differentiation in a cell autonomous environment.

By generating GC neurospheres from different mouse models we are now addressing important issues related to Hh signaling, but also to other pathways. In example, we have shown that the SmoM2 (Trp535Leu) mutation expressed in the transgenic SMOA1 medulloblastoma mouse model does not imply a full and constitutive activation of the SMO receptor, as commonly thought. Moreover, we have used these cultures to show that the knock-out of the DNA repair gene NBS1 specifically impairs Hh-dependent GCPs growth.