Final report for the ANN 125583 project

The role of activin signaling in small cell lung cancer

Small cell lung cancer (SCLC) is accounting for approximately 13%–15% of all lung cancers and it is a highly aggressive and widely metastatic malignancy with more than 200,000 new patients annually. In order to develop optimal treatment strategies and predict clinical outcome it is important to find independent prognostic factors for this invasive disease. Activin A (ActA), member of the TGF-β cytokine superfamily, has a broad range of well-known functions in physiological conditions (i.e., embryonic development, pancreatic function, bone formation and ECM deposition). Yet, its role in tumor development and progression is contradictory and depends on the tumoral entity. In addition to its contradictory effects on cell growth, apoptosis, migration, invasion and metastasis formation, ActA is capable to affect the tumor microenvironment and immune system as well. Members of activin signaling pathway have been found deregulated or mutated in many malignancies, however in SCLC the exact role of activin signaling is still unexplored.

First, to evaluate the prognostic value of ActA in SCLC patients, ActA levels were measured by ELISA in plasma samples of 79 SCLC patients and 67 age- and gender-matched healthy individuals. According to our recently published paper based on our clinical results from the subjected grant [1], SCLC patients showed significantly increased ActA levels in plasma compared to controls ($p < 0.0001$). Furthermore, extensive stage SCLC patients also had significantly higher circulating ActA levels than patients with limited stage disease ($p = 0.0179$). Moreover, when Kaplan-Meier curves were analyzed, patients with high plasma ActA concentration (dichotomized by median: 548.8 pg/ml) exhibited significantly worse median overall survival compared to those with low ($p = 0.0009$). Importantly, blood ActA levels were able to discriminate between good and poor outcome in limited and extensive disease ($p = 0.0462$ and $p = 0.0260$, respectively). Multivariate analysis revealed that circulating ActA was an independent prognostic factor in SCLC ($p = 0.023$). Altogether, ActA plasma levels should be considered during therapeutic decision-making in SCLC.

Our next aim was to investigate ActA expression on human SCLC FFPE sections. Since four different commercially available antibodies failed to be specific (IHC), we established *in situ* hybridization (ISH) to make an attempt on mRNA level (*Inhba*)

instead. All tested samples were negative however the positive control (provided by the company) was positive for *Inhba (Figure 1).*

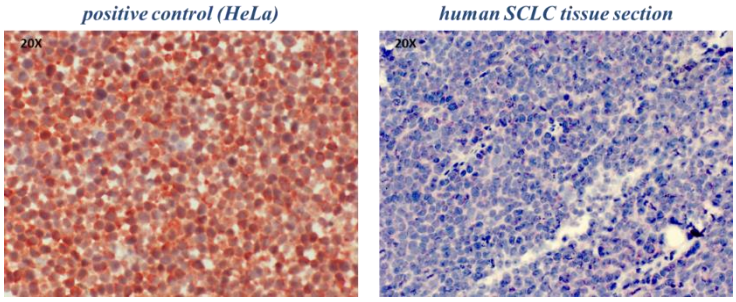

Figure 1: *In situ* hybridization failed to detect *Inhba* on human SCLC FFPE sections

To further investigate the source of ActA, we measured the ActA expression in 28 SCLC cell lines on RNA and protein levels by qPCR and ELISA, respectively. Although, all of them expressed the three ActA receptors necessary for proper signal transduction, ActA was detectable in 18 cell lines by qPCR and only 7 out of the 28 cell lines expressed ActA on protein level. Nevertheless, when cell line supernatants were analyzed, secreted ActA was present in 2 samples.

To reproduce the high circulating ActA level that we observed in human patients, we chose one cell line with high ActA secretion (SHP77) for subcutaneous inoculation into mice. Plasma samples were collected from mice bearing around 1 cm^3 s.c. tumor (n=6) and from tumor-free control mice (n=6), then ActA levels were measured by ELISA. Unfortunately, we could not demonstrate significantly elevated plasma ActA level in this model.

Although, ELISA detected ActA in 7 cells, when protein profiles of the cell lines were determined by proteomics (the proteomic analysis was performed in collaboration with the group of György Markó-Varga at Lund University), ActA was identified with low/medium confidence and therefore was excluded from further statistical analysis. The above-mentioned results (ActA was undetectable on tumor tissue; only 7 cells expressed ActA measured on protein level; none of the cells showed ActA expression according to proteomics when strict statistical conditions were adjusted; secreted ActA did not enter the bloodstream *in vivo*) suggested that the source of high circulating ActA may not the tumor tissue itself.

A review from 2019 dealing with SCLC subtypes suggested four transcriptional regulators (ASCL1, NEUROD1, POU2F3, YAP1) which could determine the expression

profile of cells within these subtypes [2]. ASCL1 (SCLC-A) and NEUROD1 (SCLC-N) are included into the earlier used so called NE-high subtype, while POU2F3 (SCLC-P) and YAP1 (SCLC-Y) are part of the NE-low subtype. Our submitted manuscript revealed that these subtypes had strong association with the prognosis of the disease [3]. We classified our 28 cell lines into subtypes by determining the expression of the four transcription factors by qPCR to investigate the association of ActA with the subtypes. The subtypes of these cell lines were verified by Western blot and proteomic analyses. Finally, our data showed that there was no correlation between ActA expression and SCLC subtypes.

To investigate the direct effects of the observed high plasma ActA level on the biological behavior of SCLC tumor cells, we transfected two tumorigenic cell lines GLC4 and Crl-2066 with ActA constructs to generate ActA overexpressing cell lines. According to our classification, GLC4 cell line represents the NE-high SCLC-N subtype whilst Crl-2066 cell line belongs to the NE-low SCLC-Y subtype. This makes possible the examination of the effect of ActA on different SCLC subtypes. qPCR, *in situ* hybridization and ELISA analyses showed elevated expression in ActA transfected cell lines compared to control lines even on the *in vivo* developed xenografts *(Figure 2).*

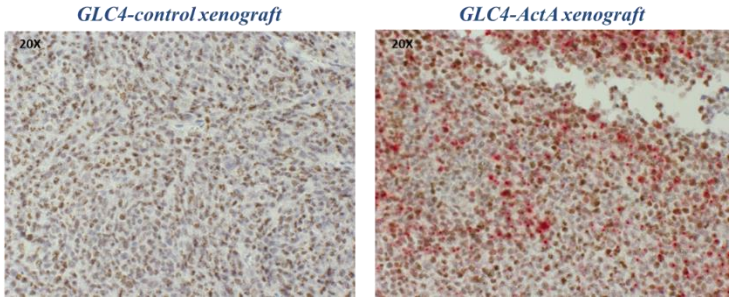

Figure 2: *In situ* hybridization confirmed stable ActA expression *in vivo*

The signal transduction pathway of these cells seemed to be active hence the Smad2 and Smad3 phosphorylation were confirmed by Western blot analysis. Therefore, GLC4 and Crl-2066 are suitable for the investigation of autocrine and paracrine effects of ActA. *In vitro* assays revealed that neither ActA treatment, nor ActA overexpression had a significant impact on viability, cell cycle distribution, proliferation, adhesion, migration and invasion.

To explore the direct effects of ActA on SCLC xenografts, angiogenic properties and connective tissue structure were investigated by immunohistochemistry. We observed

identical blood and lymph vessel density and structure when distribution of CD31, lyve-1 and laminin were examined. The labeling index of endothelial cells was also equal. Next, we stained the xenografts for several extracellular matrix components however collagen-1, α-SMA, desmin and fibronectin stainings failed to indicate any alteration in connective tissue structure.

Our next aim was to analyze the effect of ActA on the dynamics of *in vivo* tumor growth, ActA overexpressing and control cells were inoculated subcutaneously into SCID and NSG mice. When subcutaneous xenografts were investigated in immunosuppressed SCID mice (T- and B-cell deficient), GLC4-control xenografts showed growth delay compared to GLC4-ActA xenografts. This is in accordance with our clinical results where elevated ActA associated with worse prognosis. Interestingly, we observed opposite effect in the case of Crl-2066. Crl-2066-ActA xenografts showed growth delay compared to Crl-2066 controls *(Figure 3)*. In NSG mice (NK-, T- and B-cell deficient) the tumor promoting effect of ActA disappeared in the case of GLC4 cell line, the control- and ActA overexpressing xenografts grew similarly. Probably, the observed tumorigenic effect of ActA came from the attenuation of NK cell activity in SCID mice therefore it disappears in NSG mice which is lacking NK cells. On the other hand, ActA transfected Crl-2066 line showed significantly decreased growth rate in both mice model. Next, we wanted to find the reason for this antitumorigenic effect of ActA. Since the proliferation rate of Crl-2066 transfected cell lines were similar *in vitro*, these differences in Crl-2066 tumor growth should be determined by *in vivo* conditions.

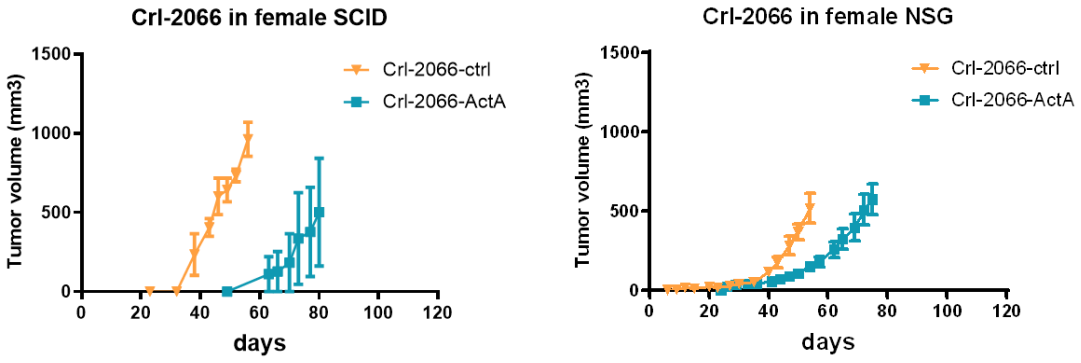

Figure 3: Crl-2066 xenograft growth

Since growth rate of Crl-2066 line was decreased in both SCID and NSG mice as mentioned above, this effect can be dependent on other members (than solely NK -cells) of the immune system. Therefore, we investigated the role of other components of the

immune system with regard to tumor progression in Crl-2066 xenografts. Short- and long-term xenografts (harvested 1-5 days and 8-11 weeks after tumor cell inoculation, respectively) were analyzed for NK cell- (CD335), macrophage- (CD68, CD86, CD163) and neutrophil granulocyte (arginase-1) infiltration by immunostaining. Investigation of long-term xenografts failed to demonstrate altered macrophage polarization (M1: CD86, M2: CD163) or difference in the localization of immune cells (only M1 macrophages infiltrated the tumor tissue), whilst in the short-term experiments ActA overexpressing xenografts showed much higher number of infiltrated antitumorigenic M1 macrophages and NK cells compared to control xenografts. The sudden increase in the number of immune cells was paralleled by very low number of living tumor cells (determined by the immunohistochemical detection of a human specific nuclear factor (SRRM2) within the lesions in the ActA overexpressing tumor mass) *(Figure 4)*. These results support the role of ActA in NK cell- and M1 macrophage mediated tumor cell killing at the very early phase of tumor development which can be responsible for the observed delay in tumor growth.

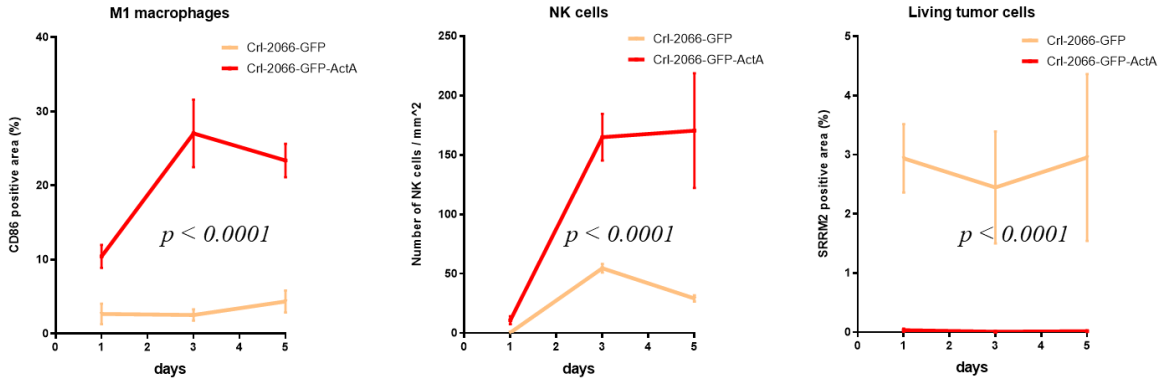

Figure 4: Crl-2066 short-term immune infiltration and number of living tumor cells

The number and distribution of neutrophil granulocytes and M2 macrophages did not differ between the control and transfected samples therefore may not have role in this process.

In the long term experiments ActA overexpressing tumors (Crl-2006) had higher tumor take in NSG mice than in SCID mice. Probably, this difference comes from the lack of NK cells in NSG mice. (Difference in tumor take could come from the initial defensive action of the immune system which in certain animals results in zero surviving tumor cells).

The delayed growth in the case of ActA overexpressing xenografts (compared to controls) in NSG mice was an NK cell independent effect and may be caused by M1 macrophages. In SCID mice the delay is caused by a concerted action of both NK cells and M1 macrophages. In summary, in spite of the initial defense activities of NK cells and M1 macrophages (which are probably responsible for the delay in tumor growth) after the tumors became established by the surviving tumor cells the NK cells and M1 macrophages are unable to further control the tumor growth.

In line with these findings, KEGG pathway analysis from proteomic data confirmed highly upregulated immune associated pathways in Crl-2066-ActA supernatant (i.e., hematopoietic cell lineage, cytokine-cytokine interaction, Th_{17} cell differentiation) *(Figure 5)*. Probably, the secreted cytokines served as direct triggers for the recruitment of immune cells which as a result led to an anti-tumorigenic effect. Proteomic data of the secreted proteins demonstrated the presence of C-reactive protein which has main roles in macrophage activation, M1 macrophage polarization and NK cell mediated cytotoxicity. Furthermore, Crl-2066-ActA cells also expressed the membrane surface protein glypican-3, which potentially labeled the tumor cells for macrophage- or NK cell mediated killing.

As mentioned above, opposite effect was found in the case of GLC4 xenografts, accordingly, ActA overexpressing subline grew out earlier in SCID mice (vs. controls). As a confirmation, proteomic analysis revealed that GLC4-ActA upregulated members of MAPK and Akt pathways for example, regulators of tumor-associated macrophage polarization, mainly in the direction of M2 tumorigenic macrophages. Nevertheless, GLC4-control cells upregulated the IL-17 signaling pathway which probably initiated an anti-tumorigenic reaction and in turn suppressed the tumor growing. To strengthen this theory additional short-term experiments are in progress.

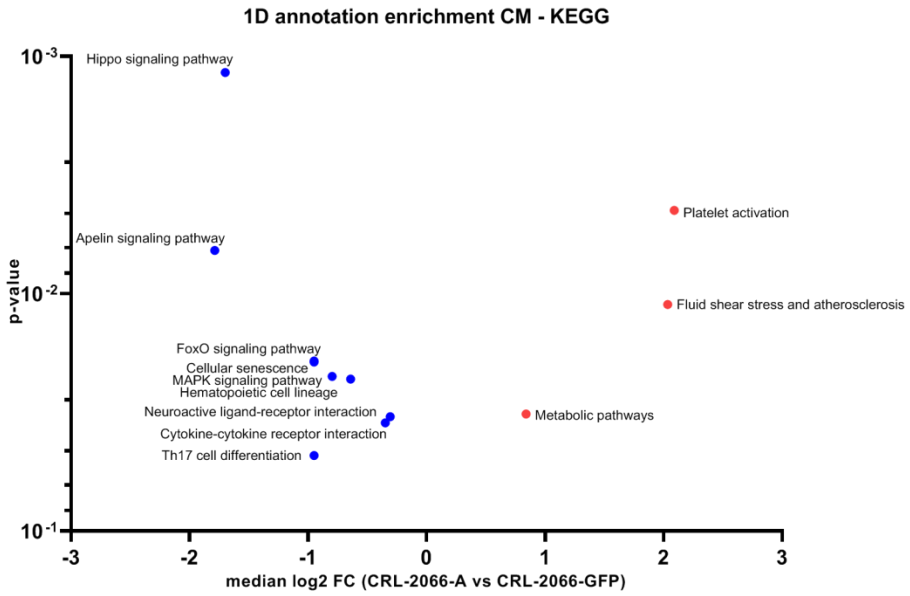

Figure 5: Crl-2066 KEGG pathway analysis*

*: As greater is the distance from zero as higher is the fold change.

(blue dots: upregulated pathways in Crl-2066-ActA supernatant;

red dots: upregulated pathways in Crl-2066-control supernatant)

According to our results, in human patients depending on the SCLC subtype ActA may trigger a defensive immune reaction against the tumor. However, this may be effective only in the early stage of tumor development and it fails as the tumor burden increases and overrides the tumor cell eliminating capacity of the immune system. The source of ActA is unknown but this factor is probably continuously and by a positive feedback increasingly produced during tumor development. This eventually manifests in the elevated plasma level of ActA.

References

1. **In this article the NKFI (National Research, Development and Innovation Office) support is indicated with the ID of the present project:** Barany N, Rozsas A, Megyesfalvi Z, Grusch M, Hegedus B, Lang C, Boettiger K, Schwendenwein A, Tisza A, Renyi-Vamos F, Schelch K, Hoetzenecker K, Hoda MA, Paku S, Laszlo V, Dome B. Clinical relevance of circulating activin A and follistatin in small cell lung cancer. Lung Cancer. 2021 Nov;161:128-135.
2. Rudin CM, Poirier JT, Byers LA, Dive C, Dowlati A, George J, Heymach JV, Johnson JE, Lehman JM, MacPherson D, Massion PP, Minna JD, Oliver TG, Quaranta V, Sage J, Thomas RK, Vakoc CR, Gazdar AF. Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data. Nat Rev Cancer. 2019 May;19(5):289-297.
3. **In this manuscript (under revision) the NKFI (National Research, Development and Innovation Office) support is indicated with the ID of the present project:** Megyesfalvi Z, Barany N, Lantos A, Valko Z, Pipek O, Lang C, Schwendenwein A, Oberndorfer F, Paku S, Ferencz B, Fillinger J, Lohinai Z, Moldvay J, Galffy G, Rezeli M, Rivard C, Hirsch F R, Bracic L, Popper H, Kern I, Kovacevic M, Skarda J, Marko-Varga G, Bogos K, Renyi-Vamos F, Hoda M A, Klikovits T, Hoetzenecker K, Schelch K, Laszlo V, Dome B. Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected SCLC: an international multicenter study. J Pathol.