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Sonic Hedgehog in cancer stem cells: a novel link with autophagy

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ABSTRACT

The Sonic Hegdehog/GLI (SHH/GLI) pathway has been extensively studied for its role in developmental and cancer biology. During early embryonic development the SHH pathway is involved mainly in pattern formation, while in latter stages its function in stem cell and progenitor proliferation becomes increasingly relevant. During postnatal development and in adult tissues, SHH/GLI promotes cell homeostasis by actively regulating gene transcription, recapitulating the function observed during normal tissue growth. In this review, we will briefly discuss the fundamental importance of SHH/GLI in tumor growth and cancer evolution and we will then provide insights into a possible novel mechanism of SHH action in cancer through autophagy modulation in cancer stem cells. Autophagy is a homeostatic mechanism that when disrupted can promote and accelerate tumor progression in both cancer cells and the stroma that harbors tumorigenesis. Understanding possible new targets for SHH signaling and its contribution to cancer through modulation of autophagy might provide better strategies in order to design combined treatments and perform clinical trials.

Key words: Cancer Stem Cells, Sonic Hedgehog, Cell Survival, Autophagy, neuroblastoma, cancer therapy

There are several developmentally expressed signaling molecules that have relevance in tumorigenesis and cancer. One of them, the hedgehog (HH) pathway, plays a key role in the regulation of embryonic development and governs processes such as cell differentiation, cell proliferation and tissue patterning. In the adult, Sonic Hedgehog, the most studied member of the vertebrate HH family, functions in tissue repair and regeneration, along with maintenance of stem cells. In adult tissues, SHH can recapitulate the gene expression that is achieved during embryogenesis through the selective activation of transcription factors. In recent years the number of identified genes that are directly regulated by the SHH pathway has increased, and can be related to oncogenic processes since several of the new targets are implicated in cancer biology (Table 1). Here, we will shortly summarize the multifaceted potential of SHH in tumor growth and maintenance according to the current literature. We will then focus on a previously unreported process controlled by SHH, autophagy, and propose an intriguing connection between a pivotal growth factor and a key cellular response as an emerging therapy that could be targeted to induce tumor cell death.

THE SHH/GLI PATHWAY AT A GLANCE

SHH is a secreted glycoprotein that activates signaling in target cells by binding to its 12-pass transmembrane receptor Patched 1 (Ptc/Ptch), which unleashes Smoothened (Smo), a seven-pass transmembrane protein G-coupled co-receptor to trigger downstream activation of the GLI family transcription factors. In mammals, canonical SHH signaling promotes localization of Smo to the primary cilium, a microtubule-based specialized cell surface protrusion, considered today a critical organizer for molecules involved in SHH signaling in vertebrates (Goetz et al., 2009, Ezratty et al., 2011). SHH ultimately exerts its effects by influencing the balance between GLI activator and repressor forms. Smo activation leads to the stabilization of GLI3 transcription factor, that, together with GLI2, act as the canonical effectors activating *gli1* and other target genes. In the absence of SHH, GLI3, and, to a lesser extent, GLI2 truncated forms can mediate in transcription acting as repressors (Aza-Blanc et al., 2000).

SHH/GLI PATHWAY IN CANCER

Altered SHH pathway activation, as revealed by up-regulation of *gli1* or *patched1* expression, has been involved in different types of solid and non-solid cancers, including glioma, medulloblastoma, neuroblastoma, leukemia, gastric cancer, and other tumors (Katoh and Katoh, 2009). Indeed, aberrant activity of SHH has been extensively connected to different aspects of cancer development, from tumorigenesis to metastasis. Accordingly, recent clinical trials with Hh pathway antagonists have validated this pathway as a promising anticancer target. Next, we will review the role of SHH in cancer pathogenesis, in particular how SHH impacts to promote and maintain malignancy from normal tissue to tumors focusing on diverse aspects of gene regulation ranging from tumor initiation, invasiveness promotion to nutrient recycling by autophagy.

INITIATION

There has been a long debate to describe the initiation cells that give rise to cancer. Even though the classification of cancers is relatively direct, the understanding of the ontogenetic origin of cancer remains elusive. One reason is that every cancer is different in terms of originating tissue, formation, and gene expression (Ezratty et al., 2011). It has been proposed that cancer initiates with a small number of "stem cells", that have the capacity to replenish the tumor in its entirety (Hill and Wu, 2009). Cancer stem-like cells (CSCs) have been identified

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TABLE 1

SHH targets associated to cancer. State-of-the-art listing of SHH-GLI controlled genes related to tumorigenesis and tumor progression

Target gene	Function in cancer	Regulation	Reference
ABCG2	ABC transporter (drug resistance, cell survival)	Direct	Singh et al., 2011(Singh et al., 2011)
Ascl1	Neurogenic bHLH transcription factor, gene regulation	Direct	(Voronova et al., 2011)
Bcl-2	Antiapoptotic protein, cell survival	Direct	(Bigelow et al., 2004)
Bmi1	Stem cell marker	Direct	(Wang et al., 2012)
BMP-2	Patterning, endochondral ossification	Direct	(Zhao et al., 2006)
Cathepsin B	Lysosomal protease	Non determined	(Hwang et al., 2009)
Cyclin D1	Cell cycle	Direct	(Hu et al., 2006)
Cyclin D2	Cell cycle	Direct	(Yoon et al., 2002; Yoon et al., 2009)
cyr61	Pro-angiogenic factor	Direct	(Harris et al., 2011)
DNMT1	Epigenetic gene regulation	Direct	(He et al., 2011)
DNMTa	Epigenetic gene regulation	Direct	(He et al., 2011)
DR4	TRAIL induced death receptor	Direct	(Kurita et al., 2010)
Fbn2	Microfibrills component	Direct	(Yu et al., 2009)
Fgf15	CNS development	Direct	(Komada et al., 2008)
FOXA2 (HNF3β)	Transcriptional activator in liver	Direct	(Sasaki et al., 1997)
FoxF1	Mesenchymal transcription factor and potential tumor suppressor	Direct	(Madison et al., 2009)
FoxL1	Mesenchymal transcription factor	Direct	(Madison et al., 2009)
Hes1	Transcriptional repressor, Notch target	Direct	(Wall et al., 2009)
Hhip	Hedgehog interacting protein	Direct	(Vokes et al., 2007)
Hrt3	Notch target	Indirect	(Morrow et al., 2009)
IGFBP3	Igf binding protein	Direct	(Yu et al., 2009)
IGFBP6	Igf binding protein	Direct	(Yu et al., 2009)
K17	Epithelia development, EMT	Direct	(Bianchi et al., 2005)
KLK2	Serine protease	Non determined	(Chen et al., 2010)
KLK3	Serine protease	Non determined	(Chen et al., 2010)
Krox-20	Associated with desmoplastic medulloblastoma	Direct	(Yoon et al., 2009)
MEF2C	Myogenesis and angiogenesis	Direct	(Voronova et al., 2011)
Mycn	Oncogene	Direct	(Hu et al., 2006)
Myf5	Muscle differentiation	Direct	(Gustafsson et al., 2002)
Nkx2.1	Potential oncogene	Direct	(Vokes et al., 2007)
Nkx2.2	Potential oncogene	Direct	(Vokes et al., 2007)
Osteopontin	EMT regulation	Direct	(Yoon et al., 2002; Das et al., 2009)
Pax2	Tumor suppressor target gene	Direct	(Hu et al., 2006)
Plakoglobin	Catenin-cadherin complex, EMT	Direct	(Yoon et al., 2002)
Ptch1	Catenin-cadherin complex, EMT	Direct	(Yoon et al., 2002)
	Negative regulator of Hh	Direct	(Alexandre et al., 1996)
Ptch2	Patched homolog	Direct	(Vokes et al., 2007)
RegIV	Multiple functions in cancer	Direct	(Wang et al., 2011)
RGS4	G protein regulator	Direct	(Yu et al., 2009)
Sall1	Transcription factor involved in tumorigenesis	Direct	(Hu et al., 2006)
Sox9	Transcription factor involved in development and oncogenesis	Direct	(Bien-Willner et al., 2007)
Stathmin 1	Microtubule dynamic-regulating oncoprotein	Non determined	(Chung et al., 2010)

in solid tumors of the breast, colon, brain and other sites. They can differentiate into all the cell phenotypes of the parental tumor. This developmental scheme has been demonstrated for many cancer types, including neural (Hemmati et al., 2003) and non-neural tumors (Richardson et al., 2004, O'Brien et al., 2007). The origin of CSCs is not fully understood, but data suggest that they originate from normal stem or progenitor cells, or possibly other cancer cells. These cells are capable to self-renewal, to give rise the different tumor cell types, and to maintain tumor growth. Other key features include activation of pluripotency genes (Oct4, Sox2, Nanog), formation of tumor spheres in low-adherence cultures, and multi-drug resistance. CSCs can be identified by distinct markers, including the cell surface marker CD133 (also known as prominin 1), BMI1 and CD44 (Neuzil et al., 2007) [Figure 1 and Table 1]. Noteworthy, these cells have been shown as resistant to cancer therapies and CSCs have therefore been proposed to be the cells of origin for tumor relapse. Thus, while the transcriptome of CSCs may not fully match that of the cognate stem cells, pluripotent tumor cells with stem cell phenotype and capacity probably contribute significantly to the phenotypic heterogeneity seen in cancers.

CSCs use a variety of signaling pathways to undergo self-renewal and differentiation, including Wnt, Notch, and HH (Barker and Clevers, 2006; Wang et al., 2012a). There is increasing evidence that connects the SHH/GLI pathway and tumor initiation specific markers as targets (Katoh and Katoh, 2009). In neuroblastoma, for instance, a SHH pathway pharmacological loss of function reduces a CD133/CD15 positive compartment (Schiapparelli et al., 2011). In medulloblastoma, stem cell markers have been involved in SHH tumor propagation (Read et al., 2009), and are also directly controlled by the pathway (Wang et al., 2012). Thus, the SHH/GLI pathway may have different roles during cancer initiation, activating in cells with tumorigenic potential, and up-regulating genes that are involved in cell "stemness" maintenance.

GROWTH

The SHH pathway has been classically involved in growth during embryonic development, controlling key genes that modulate cell proliferation such as *cyclins* and *n-myc* (Figure 1). Abnormal SHH signaling activity during cancer has similarities to normal development and organ growth; tumor cells recapitulate development, but aberrantly. Gain of function mutations in key components of the SHH pathway, such as Patched 1 or Smo, are sufficient to generate tumors in different tissues such as skin, cerebellum and prostate (Athar et al., 2006,Yang et al., 2008, Sanchez et al., 2004). SHH controls genes (Mill et al., 2005), both directly and indirectly, that amplify a secondary response signal, since many of these target genes control their own multiple targets (Eilers and Eisenman, 2008).

MAINTENANCE

During embryonic development and in normal tissue homeostasis, SHH is involved in progenitor cell maintenance and has also been shown to act as a survival factor in different

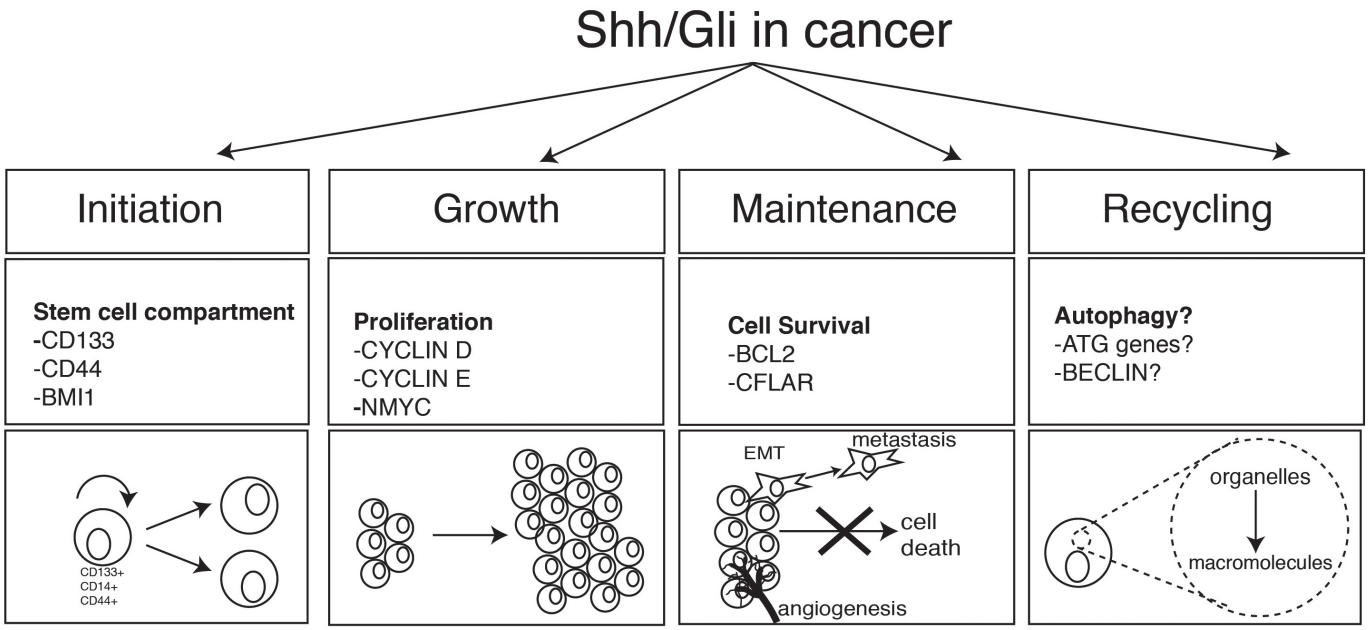

Figure 1: SHH functions in cancer. The canonical SHH pathway has been related to many aspects of tumorigenesis and tumor progression. During cancer initiation, there is a SHH/GLI dependent up-regulation of the CSC marker BMI1, sharing expression with CD15 or CD133. SHH is also involved in maintaining the stem cell niche responsible for tumor initiation. Later on, during tumor growth SHH/GLI controls transcription of genes implicated in cell proliferation and cell cycle progression. In order to maintain tumor size and support pro-invasive processes, SHH pathway activates cell survival, EMT transition, angiogenesis and metastatic mechanisms through extensive direct gene regulation. SHH is also acting indirectly related to other signaling pathways. We propose that SHH could be involved in the autophagic process as a way to recycle nutrients, promoting cell survival and resistance to adverse environments. All these functions highlight the importance of the SHH pathway in cancer biology and justify its use as a strategic target for combined pharmacologic treatments.

tissues (Krüger et al., 2001; Machold et al., 2003). In cancer progression, both processes are deregulated, implicating SHH dysfunction. In order to maintain the number of cells growing in a tumor, SHH can control cell death avoidance/survival. Canonical SHH signaling positively controls *bcl-2* (Bigelow et al., 2004), an important regulator of apoptosis and proto-oncogene that has been related to cell survival in multiple solid and non-solid cancers. SHH also modulates apoptosis induced by the Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through repression by GLI3, binding the cognate death receptor-4 (DR4) promoter (Kurita et al., 2010).

ANGIOGENESIS

The term angiogenesis literally means "new blood vessel formation", and performs a critical role for cancer progression that facilitates tumor growth and survival. Angiogenesis enables tumor expansion, local expansion and dissemination (Bergers and Benjamin, 2003). It is a well-regulated process, driven by specific pro-angiogenic factors and extracellular proteins expressed by endothelial cells (Schmidt et al., 2007). Pro-angiogenic factors such as cyr61, VEGF, neuropilin-1 and CD24 have recently been shown as regulated by the canonical SHH pathway. SHH appears to play a critical part in the biology of the perivascular niche and has been implicated in vascular formation and function within the tumor (Harris et al., 2011; Cao et al., 2012, Geng et al., 2007). Of note, the angiogenic process is closely related to other massive changes in cancer cells, in order to promote colonization of new niches.

EPITHELIAL MESENCHYMAL TRANSITION (EMT) AND METASTASIS

EMT refers to a cellular reorganization process that is key to embryonic development. It results in down-regulation of cell adhesive mechanisms, loss of cell polarity, and gaining of invasive and migratory mesenchymal properties. The EMT series of events also occur during tumorigenesis, allowing tumor-initiating cells to metastasize. During EMT, cells down-regulate E-cadherin, a membrane-bound glycoprotein involved in the adherence of adjacent cells. The loss of E-cadherin in primary tumor tissue has been linked to tumor metastasis and poor prognosis. SHH is involved in EMT along with Notch and BMP signaling pathways and other niche factors, such as members of the transforming growth factor-β (TGF-β) superfamily of cytokines (Bailey et al., 2007; Yoo et al., 2011). These different pathways are also connected, since SHH for instance, directly upregulates Jagged2 to activate the Notch pathway response (Kasper et al., 2006). SHH also regulates the expression of other proteins involved in EMT and metastasis as Snail (Wanshura et al., 2011) and the down-regulation of E-cadherin, contributing to the EMT and metastatic phenotype (Maitah et al., 2011).

AUTOPHAGY AS A MECHANISM FOR SELECTIVE TUMOR GROWTH

Tumor cells often acquire the ability to evade death by inactivating survival pathways that normally function to eliminate damaged and harmful cells. A strictly regulated mechanism that achieves this removal and reutilization is autophagy. Autophagy is generally thought to play a pro-survival role and can be up-regulated in response to both external and intracellular factors, including amino acid starvation, growth factor withdrawal, low cellular energy levels, endoplasmic reticulum (ER) stress, hypoxia, oxidative stress, pathogen infection, and organelle damage (Janku et al., 2011). It is therefore considered a self-defense mechanism, where macromolecules and complete organelles are engulfed in perinuclear double membrane vesicles and degraded in lysosomes (Mizushima, 2007;Chen and White, 2011).

The role of autophagy in cancer is complex, depending mainly on the tumor stage. It has been proposed that autophagy may have a dual role, as a tumor suppressor in normal cells by degrading oncoproteins and, later, allowing cancer cells to survive during metabolic stress (Janku et al., 2011). In this sense, although autophagy is a mechanism of tumor suppression, it also confers stress tolerance that enables tumor cells to survive under adverse conditions by recycling of nutrients for metabolic needs, a fundamental aspect of tumor progression. It has been observed that extensive autophagy is generated by tumor hypoxia and anaerobic glycolysis, whereas angiogenesis maintains low autophagic activity. In fact, autophagy localizes to hypoxic regions of tumors most distal to blood vessels where is supports tumor cell survival (Sivridis et al., 2011).

Autophagy inhibition may result an interesting strategy for pharmacological studies in order to limit tumor nutrient availability and energy demands. The successful development and application of autophagy regulators is important. Signaling pathways that promote autophagy are therefore potential candidates for inhibitor development.

A NEW PARADIGM FOR SHH/GLI THERAPEUTIC ACTION: INHIBITION OF AUTOPHAGY WITH HH INHIBITORS

To date, a relationship between the SHH and autophagy pathways has not been reported, although it has been shown that there are cancers that are sensitive to both pathways, as for instance neuroblastoma (Mao et al., 2009; Mohan et al., 2011; Xu et al., 2012).

In order to shed light on a possible link between SHH and autophagy we tested the effect of a pharmacological loss of function for the SHH pathway in the number of LC3 [microtubule-associated protein light chain 3] positive vesicles, using the neuroblastoma cell line SHSY5Y (Biedler et al., 1978). To date, the detection of processed LC3 by western blot or fluorescence studies, together with electron microscopy for autophagosome formation, have been the mainstays for autophagy detection (Lazova et al., 2010). Cyclopamine (cyc), an alkaloid that functions as SHH antagonist, with several derivatives under clinical trials, was used under a nutrient starvation protocol consisting of serum starvation (Allison, 2012). Two hours of cyc pretreatment of SHSY5Y cultures prevented LC3 vesicles formation (Figure 2A-B). This reduction in LC3 autophagosome positive cells was accompanied with increased Caspase 3 positive cells, suggesting that these cells underwent apoptosis, probably also related to genes controlled by SHH/GLI (Figure 2D-H). To evaluate if the SHH pathway controls essential autophagic genes such as *atg5* or *beclin1 (bcn1)*, we used a SHH-sensitive cell line, C3H10T1/2, treated with SHH-N conditioned media or control (Δ64-SHH) and evaluated *atg5* and *bcn1* levels. Pathway activation was monitored by *ptc1*. Of note, *atg5* decreased in

Δ64-SHH treated cells, whereas *bcn1* did not change under these conditions. This suggests that autophagy regulation by SHH could be driven by a transcripcional control of specific key autophagic genes. Importantly, using a yeast-reverse one-hybrid system (Milla et al., 2012) and bioinformatics we were able to detect non -consensus GLI binding sites (GBS) in the first and seventh intron of the mouse *atg5*. We also found multiple GBS in the human ATG5 promoter (unpublished

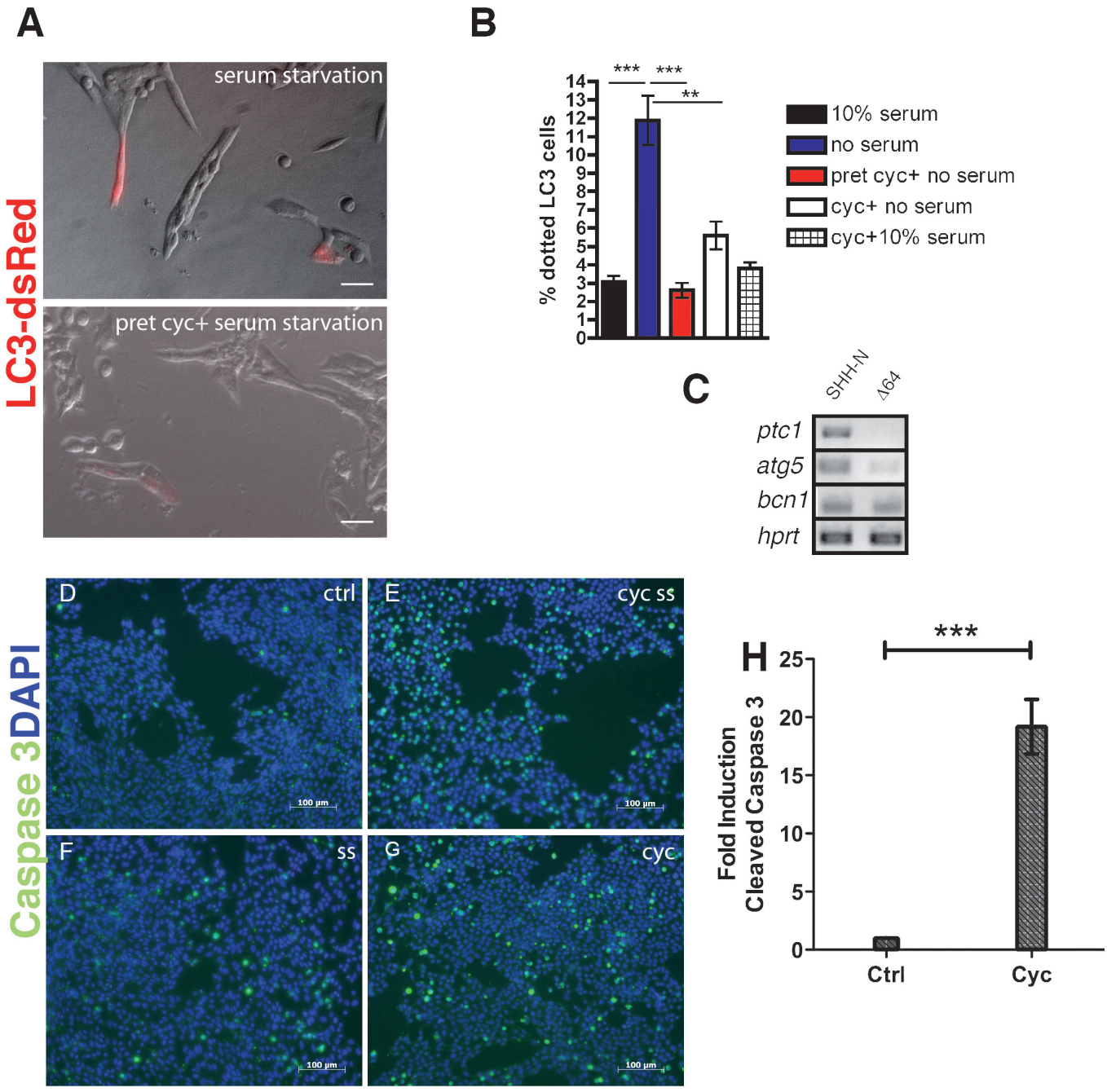

Figure 2: SHH signaling regulates autophagy in the neuroblastoma cell line SHSY5Y. 6-hour serum-starved cells increase dotted LC3-DsRed positive cells in comparison to 10% fetal bovine serum cultured controls, suggesting increased autophagy (A-B). Treatment with 10μM cyc significantly decreases the number of LC3-dotted cells compared to the control serum-starved condition. Strikingly, the decrease in LC3 positive cells after a 2-hour cyc pre-treatment is even more pronounced (B). Representative images of replicates of 4 experiments are shown in upper and lower panel. Bar=50 μm. (C) The SHH pathway reporter cell line C3H10T1/2 were treated for 24 hours with conditioned media obtained following 48-hour transfection of C3H10T1/2 cells with either SHH-N or Δ64-SHH, a mutant form of Shh, which is unable to signal. Pathway activation was monitored by *ptc1*. Note the *atg5* decrease in Δ64-SHH treated cells. The *bcn1* autophagic gene does not change under these conditions. (D-G), Cyc treatment drives cells to apoptosis as evidenced by an increased number of cleaved caspase 3-positive cells $p<0.0006$. Quantitation in (H). Each bar in the graph represents the average of separate triplicate determinations showing the standard deviation of the mean. P value $p<0.001$.

results). These data suggest the attractive possibility that SHH pathway might control the autophagic series of events in cancer cells. From a therapeutic point of view, it would be interesting to evaluate the effect of combined anticancer pharmacological antiautophagic agents and Hh pathway inhibition on tumor cell survival. SHH pathway inhibition could act as a novel sensitizer to increase efficiency of conventional chemotherapeutic agents in cancer by inducing apoptosis. Consistent with our findings, previous reports have suggested that several cytotoxic chemotherapeutic agents induce autophagy, and inhibition of autophagy enhanced their efficiency *in vitro* (Guo et al., 2012). Combinations of other anticancer drugs with autophagy inhibitors have also shown success in preclinical models (Amaravadi et al., 2007, Carew et al., 2010).

Autophagy could also act in the cells that fuel the tumor, especially in the early stages, modulating the tumor stroma or the "stem cell niche". Tumor cells exploit the surrounding stromal environment through the recruitment of these nonmalignant cells that provide physiological resources to facilitate tumor progression. It has been proposed that the stromal cells increase autophagy in order to speed their metabolism and generate anaerobic extra-mitochondrial glycolisis, allowing them to boost their energy production and oxidative stress, accelerating a random mutagenesis in cancer cells (Lisanti et al., 2010). We only analyzed the changes in neuroblastoma cells produced by the SHH pathway, it is important, however, to conceive these changes as cooperative and strongly related to the tumor microenvironment, occurring in parallel during each tumor stage. Experiments conducted using co-cultures, as a model, would help to understand this relationship and shed light on the *in vivo* situation. Even though autophagy plays a similar role in tumor cells as it does in normal cells, tumor cells face more stress and the dependence on autophagy may be more substantial. This differential response between normal and tumor cells in autophagy dependence may be useful for exploiting autophagy modulation in cancer therapy. In tumor initiation, for example, autophagy should be studied in the tumor-initiating cells and in the tumor niche, the stroma, in an independent manner.

Cross talk between independent yet intertwined signaling pathways of metabolism and cancer is currently a topic of intense research. Of note, SHH has recently been proposed as a general positive metabolic regulator in cancer (Bhatia et al., 2012). This observation, combined with our data indicating a SHH mediated autophagic activation, could influence cancer cells to survive and proliferate. More elaborate analysis is needed to determine if the SHH and autophagic pathways activation are coupled in different cancer types.

CONCLUDING REMARKS

Induction of cell death and inhibition of growth are the main targets of cancer therapy. In this short paper, we summarized new insights into molecular mechanisms of SHH action in cancer with special focus on autophagy. Understanding the role of autophagy in cancer treatment is critical since many anticancer therapies activate autophagy, possibly limiting their therapeutic efficacy. Here we propose that autophagy could be connected with the HH signaling pathway. The nature of this relation is of interest for the design of anticancer combined therapies, with HH and autophagy antagonists. Elucidating the interplay between autophagy, tumor cell metabolism and SHH/GLI will provide unique opportunities to identify new therapeutic targets and develop synthetically lethal treatment strategies that preferentially target cancer cells, while sparing normal tissues.

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