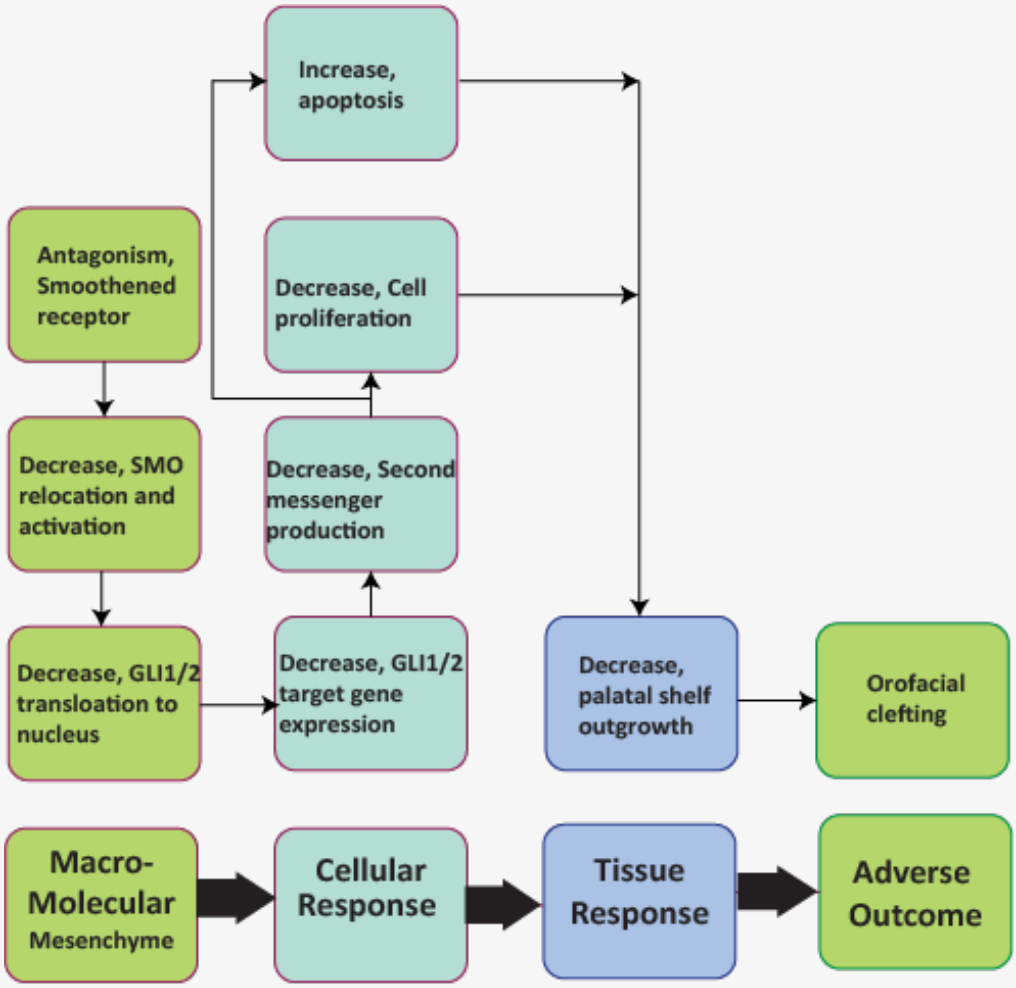
AOP ID and Title:

AOP 460: Antagonism of Smoothened receptor leading to orofacial clefting
Short Title: Anatagonsim SMO leads to OFC

Graphical Representation



Authors

Jacob I. Reynolds[1] , Brian P. Johnson[1,2]

[1]Department of Biomedical Engineering, Institute for Quantitative Health Science and Engineering, Michigan State University, East Lansing, MI

[2]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			

Abstract

The Sonic Hedgehog (SHH) is a major signaling pathway of intercellular signaling during embryonic development. Disruption of SHH during critical periods of development can lead to orofacial clefts (OFCs). In canonical SHH signaling, the SHH ligand binds to the Patched1 (PTCH1) receptor and relieves its' suppression of Smoothened (SMO) receptor. Antagonism of SMO results in disruption the downstream SHH signaling cascade. Disruption to the signaling cascade causes a decrease in the translocation of the GLI1/2 transcription factors to the nucleus resulting in a decrease in expression of the GLI1/2 target genes. This decrease in gene expression which causes a reduction in production of SHH secondary messengers, namely Fgf10 and members of the BMP family. This reduction in secondary messengers leads to an increase in cellular apoptosis and a decrease in cellular proliferation in the palatal. These events both lead to a decrease in palatal shelf outgrowth which ultimately results in a cleft. This AOP is intended to serve as a tool for risk assessment for drug and chemical exposures during embryonic development when disruption to SHH through antagonism of SMO occurs.

Background

Orofacial clefts (OFCs), encompassing cleft lip with or without palate (CL/P), and cleft palate only (CPO) represent the second most common birth defect in humans with a prevalence of 1-2/1,000 births (Lidral, Moreno et al. 2008). The etiology of OFCs is complex with approximately 50% of CPO and 70% of CL/P considered non-syndromic (2011). SHH signaling is required for normal facial development and plays a critical role in the growth of the facial processes that form the upper palate and lip (Bush and Jiang 2012, Kurosaka 2015). The epithelial derived SHH drives orofacial development through an induced gradient in the underlying mesenchyme (Lan and Jiang 2009, Kurosaka 2015). This gradient of SHH induces cellular proliferation and outgrowth of the mesenchyme (Lan and Jiang 2009). The SHH pathway is sensitive to chemical disruption and can be disrupted at multiple places along the signaling cascade during critical windows for exposure and has been shown to cause OFCs (Lipinski and Bushman 2010, Heyne, Melberg et al. 2015). The targets of this disruption include ligand modification, ligand secretion, downstream sensing, and signal transduction (Jeong and McMahon 2002, Lauth, Bergström et al. 2007, Petrova, Rios-Esteves et al. 2013). Chemical modulators of the SHH pathway have been identified including the natural alkaloid cyclopamine, both natural and synthetic pharmaceuticals, and a chemical commonly found in pesticides (Lipinski, Dengler et al. 2007, Lipinski, Song et al. 2010, Wang, Lu et al. 2012, Everson, Sun et al. 2019, Rivera-González, Beames et al. 2021).

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	2027	Antagonism, Smoothened receptor	Antagonism Smoothened
	KE	2044	Decrease, Smoothend relocation and activation	Decrease, SMO relocation
2	KE	2028	Decrease, GLI1/2 translocation to nucleus	Decrease, GLI1/2 translocation
	KE	2040	Decrease, GLI1/2 target gene expression	Decrease, GLI1/2 target gene expression
	KE	2043	Decrease, Sonic Hedgehog second messenger production	Decrease, SHH second messenger production
	KE	1821	Decrease, Cell proliferation	Decrease, Cell proliferation
	KE	1262	Apoptosis	Apoptosis
	KE	2041	Decrease, palatal shelf outgrowth	Decrease, outgrowth
	AO	2042	Ororofacial clefting	OFC

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Antagonism, Smoothened receptor	adjacent	Decrease, Smoothend relocation and activation	Moderate	Low
Decrease, Smoothend relocation and activation	adjacent	Decrease, GLI1/2 translocation to nucleus	Moderate	Low
Decrease, GLI1/2 translocation to nucleus	adjacent	Decrease, GLI1/2 target gene expression	Low	Low
Decrease, GLI1/2 target gene expression	adjacent	Decrease, Sonic Hedgehog second messenger production	Low	Low
Decrease, GLI1/2 target gene expression	adjacent	Decrease, Cell proliferation	Low	Low
Decrease, Cell proliferation	adjacent	Decrease, palatal shelf outgrowth		
Decrease, palatal shelf outgrowth	adjacent	Ororofacial clefting		

Stressors

Name	Evidence
Vismodegib	High

Name	Evidence
Cyclopamine	
SANT-1	
SANT-2	
SANT-3	
SANT-4	

Vismodegib

Vismodegib (GDC-0449) is small molecule modulator of the sonic hedgehog (shh) pathway. It functions as an antagonist by binding to Smoothened (SMO) blockings its' activation and subsequent downstream signalling cascade. Vismodegib became the first agent approved to target the shh pathway in Jan. 2012 by the US FDA. It was approved by the European Medicines Agency (EMA) in July 2012 (Meiss, Andrlová et al. 2018). It has been used to identify critical periods of development for the shh pathway. Pregnant C57BL/6J mice dosed with 40mg/kg of Vismodegib between E7 and E10.0 had a peak incidence of CPO (34.38%) at E9.5(Heyne, Melberg et al. 2015). Pregnant C57/BL6J mice treated with 100mg/kg vismodegib via oral gavage at E10.5 and E12.5 displayed a 100% penetrance of complete cleft palate (Zhang, Wang et al. 2017). In a HWJSC/HPEKp spheroid fusion model 10µm vismodegib did not affect HPEKp viability or migration, did not affect *in vitro* fusion (Belair, Wolf et al. 2018).

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus		NCBI
human	Homo sapiens	Low	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

References

Appendix 1

List of MIEs in this AOP

Event: 2027: Antagonism, Smoothened receptor

Short Name: Antagonism Smoothened

Key Event Component

Process	Object	Action
regulation of receptor activity	smoothened	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
mesenchymal cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- SMO is present in both male and females and differences in activation or antagonism between sex have not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development. Aberrant activation of HH signalling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic- SMO is conserved in both vertebrates and invertebrates. SMO signaling is dependent upon its relocation to a subcellular location. This occurs in the plasma membrane for flies (Denef, Neubüser et al. 2000) and the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005).

Key Event Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This occurs in the plasma membrane for flies (Denef, Neubüser et al. 2000) and the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005).

In the absence of Hedgehog (HH) ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO is able to relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000). This signaling to effectors results in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression(Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). The exact mechanism through which PTCH and SMO interact is not known.

An endogenous ligand for SMO has not been discovered although evidence for one exists and that PTCH controls SMO by controlling its' availability or accessibility. To support this, it has been shown that PTCH and SMO do not physically interact (Chen and Struhl 1998). PTCH acts catalytically with SMO with one PTCH receptor capable of controlling many (~50) SMO receptors (Taipale, Cooper et al. 2002). Since PTCH includes a sterol sensing domain and shares characteristics of ancient bacterial transporters, a model of PTCH functioning by pumping a sterol-like MSO regulator has been proposed (Mukhopadhyay and Rohatgi 2014). SMO is constitutively active in the absence of PTCH suggesting that the elusive molecule is an agonist (Rohatgi and Scott 2007). Conversely, the discovery that oxysterols bind to the CRD binding domain acting as positive modulators suggest that the molecule could be an agonist with PTCH functioning to sequester away or limit cellular concentration (Corcoran and Scott 2006, Nachtergaele, Mydock et al. 2012)

The activity of SMO is controlled by ligand binding (Kobilka 2007). Two separate binding pockets, one in the groove of the extracellular CRD and the other in the helices of the TMD have been identified (Nachtergaele, Mydock et al. 2012, Rana, Carroll et al. 2013, Wang, Wu et al. 2013, Byrne, Sircar et al. 2016, Huang, Zheng et al. 2018). These two binding pockets have been shown to interact in an allosteric manner (Nachtergaele, Mydock et al. 2012). The binding pocket in the helices of the TMD binds several SMO agonists including SAG as well as antagonists Vismodegib and Sonidegib. The CRD binding pocket binds cholesterol and its' oxidized derivates (Byrne, Luchetti et al. 2018). The antagonist cyclopamine binds to the TMD binding pocket and inhibits SHH signal transduction. However, in mSMO carrying the mutations D477G/E552K that disable the TMD binding pocket, cyclopamine binds to the CRD pocket and activates the pathway (Huang, Nedelcu et al. 2016). To date several oxysterols including 20(S)-hydroxylcholesterol, 22(S)-hydroxylcholesterol, 7-keto-25-hydroxylcholesterol and 7-keto-27-

hydroxylcholesterol have been identified as activators of SMO (Dwyer, Sever et al. 2007, Nachtergaele, Mydock et al. 2012, Myers, Sever et al. 2013). A binding site for 24(S),25-epoxycholesterol has been identified in the TMD pocket using cryo-EM of SMO in complex with 24(S),25-epoxycholesterol (Qi, Liu et al. 2019).

How it is Measured or Detected

Verification of binding and affinity for SMO can be measured using fluorescence binding assays and photoaffinity labeling respectively (Chen, Taipale et al. 2002). qRT-PCR can be used to determine the expression level of SMO (Lou, Li et al. 2020).

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List of Key Events in the AOP

Event: 2044: Decrease, Smoothend relocation and activation

Short Name: Decrease, SMO relocation

Key Event Component

Process	Object	Action
protein localization to cilium	smoothened	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell
Cell term

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- SMO and cilia are present in both male and females and differences in gene expression has not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development.
- Taxonomic-SMO relocation to the tip of primary cilia occurs in vertebrates Huangfu and Anderson 2005)

Key Event Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

In the absence of SHH ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000, Rohatgi and Scott 2007). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). The exact mechanism through which PTCH and SMO interact is not known.

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

How it is Measured or Detected

- Fluorescent proteins can be used tag SMO, cilia and the plasma membrane to determine if SMO has relocated to the cilia (Filipova, Diaz Garcia et al. 2020).
- Fluorescent binding assay can be used to verify if a compound binds to SMO (Chen, Taipale et al. 2002).
- Cell lines can be engineered to express Myc-tagged SMO. This gives a user friendly readout of SMO activation. (Corbit, Aanstad et al. 2005).

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Event: 2028: Decrease, GLI1/2 translocation to nucleus

Short Name: Decrease, GLI1/2 translocation

Key Event Component

Process	Object	Action
protein import into nucleus, translocation	zinc finger protein GLI1	decreased
protein import into nucleus, translocation	zinc finger protein GLI2	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

cell

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- The Gli family of transcription factors is present in both male and females and differences in activation or antagonism between sex have not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development. Aberrant activation of HH signalling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic-HH signalling including the Gli transcription factors is present in vertebrates and some invertebrates inclubind flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009).

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to a increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016).

How it is Measured or Detected

- A nuclear translocation assay (NTA) can be applied to determine the amount of protein that translocate into the nucleus (Dixon and Lim 2010).
- Nuclear protein extracts can be analysed to determine if the protein of interest (GLI1/2) translocated to the nucleus (Kim, Kato et al. 2009).
- Immunofluorescence and microscopy can be used to determine how much of a protein has translocated to the nucleus. Primary antibodies can be used to tag GLI in combination with a secondary stain for the nucleus (Blotta, Jakubikova et al. 2012).

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Event: 2040: Decrease, GLI1/2 target gene expression

Short Name: Decrease, GLI1/2 target gene expression

Key Event Component

Process	Object	Action
gene expression	zinc finger protein GLI1	decreased
gene expression	zinc finger protein GLI2	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- The GLI family of transcription factors is present in both male and females and differences in gene expression has not been demonstrated.

- Life stages- The Hedgehog pathway is a major pathway in embryonic development. Aberrant activation of HH signalling is known to cause cancer (Dahmane, Lee et al. 1997, Kimura, Stephen et al. 2005). For these reasons all stages of life are of relevance.
- Taxonomic-HH signalling including the GLI transcription factors is present in vertebrates and some invertebrates including flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

The Glioma-associated onocogene (GLI) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009). Following translocation into the nucleus, the GLI family of transcription factors initiates transcription of a variety of genes. The genes transcribed by activation of the SHH pathway are cell type dependent but commonly include GLI1 and PTCH1 (Stamataki, Ulloa et al. 2005, Cohen, Kicheva et al. 2015, Tickle and Towers 2017). During development of the neural tube SHH is associated with NKX6.1, OLIG2, NKX2.2 and the FOXA2 genes (Vokes, Ji et al. 2007, Kutejova, Sasai et al. 2016). Other genes have are known targets of GLI transcription include PTCH2, HHIP1, MYCN, CCND1, CCND2, BCL2, CFLA, FOXF1, FOXFL1, PRDM1, JAG2, GREM1, FOXB2, FOXA2, FOXB2, FOXC1, FOXC2, FOXD1, FOXE1, FOXF1, FOXF2, FOXL1 and follistatin (Katoh and Katoh 2009, Everson, Fink et al. 2017).

How it is Measured or Detected

- Changes in gene expression can be measured using serial analysis of gene expression (SAGE), rapid analysis of gene expression (RAGE), RT-PCR, Northern/Southern blotting, differential display, and DNA microarray assay.

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Event: 2043: Decrease, Sonic Hedgehog second messenger production

Short Name: Decrease, SHH second messenger production

Key Event Component

Process	Object	Action
second-messenger-mediated signaling		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- Secondary messenger production of the SHH pathway is present in both male and females and differences in gene expression has not been demonstrated.
- Life stages- The Hedgehog pathway is a major pathway in embryonic development.
- Taxonomic-HH signalling, and its' secondary messenger production is present in vertebrates and some invertebrates including flies (Denef, Neubüser et al. 2000, Huangfu and Anderson 2005)

Key Event Description

During normal Sonic Hedgehog (SHH) signaling, GLI target gene expression regulates several other signaling pathways. Expression of FOXF1 and FOXL1 upregulate BMP4, BMP 2, and FGF10 in the mesenchyme (Katoh and Katoh 2009, Lan and Jiang 2009). Induction of FGF10 in the mesenchyme is able to induce SHH in the adjacent epithelium via a positive feedback loop with FGFR2 (Cobourne and Green 2012). SHH signaling also upregulates BCL2 and CFLAR to promote cell survival (Katoh and Katoh 2009).

How it is Measured or Detected

- Changes in gene expression can be measured using serial analysis of gene expression (SAGE), rapid analysis of gene expression (RAGE), RT-PCR, Northern/Southern blotting, differential display, and DNA microarray assay.
- Antibody staining of tissue sections can be used to determine location and amounts of BMP4, BMP2, FGF10

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Event: 1821: Decrease, Cell proliferation

Short Name: Decrease, Cell proliferation

Key Event Component

Process	Object	Action
cell proliferation	cell	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation	KeyEvent
Aop:290 - Mitochondrial ATP synthase antagonism leading to growth inhibition (1)	KeyEvent
Aop:286 - Mitochondrial complex III antagonism leading to growth inhibition (1)	KeyEvent
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition via glucose depletion	KeyEvent

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone

Name
Pentachlorophenol
Triclosan
Emodin
Malonoben

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general applicable to all eukaryotes, as most organisms are known to use cell proliferation to achieve growth.

Life stage applicability domain

This key event is in general applicable to all life stages. As cell proliferation not only occurs in developing organisms, but also in adults.

Sex applicability domain

This key event is sex-unspecific, as both genders use the same cell proliferation mechanisms.

Key Event Description

Decreased cell proliferation describes the outcome of reduced cell division and cell growth. Cell proliferation is considered the main mechanism of tissue and organismal growth (Conlon 1999). Decreased cell proliferation has been associated with abnormal growth-factor signaling and cellular energy depletion (DeBerardinis 2008).

How it is Measured or Detected

Multiple types of *in vitro* bioassays can be used to measure this key event:

- ToxCast high-throughput screening bioassays such as “BSK_3C_Proliferation”, “BSK_CASM3C_Proliferation” and “BSK_SAg_Proliferation” can be used to measure cell proliferation status.
- Commercially available methods such as the well-established 5-bromo-2’-deoxyuridine (BrdU) (Raza 1985; Muir 1990) or 5-ethynyl-2’-deoxyuridine (EdU) assay. Both assays measure DNA synthesis in dividing cells to indicate proliferation status.

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Event: 1262: Apoptosis

Short Name: Apoptosis

Key Event Component

Process	Object	Action
apoptotic process		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:205 - AOP from chemical insult to cell death	AdverseOutcome
Aop:207 - NADPH oxidase and P38 MAPK activation leading to reproductive failure in Caenorhabditis elegans	KeyEvent
Aop:212 - Histone deacetylase inhibition leading to testicular atrophy	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:439 - Activation of the AhR leading to breast cancer	KeyEvent
Aop:452 - Adverse outcome pathway of PM-induced respiratory toxicity	KeyEvent
Aop:393 - AOP for thyroid disorder caused by triphenyl phosphate	KeyEvent
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent
Aop:476 - Adverse Outcome Pathways diagram related to PBDEs associated male reproductive toxicity	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

cell

Organ term

Organ term

organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Caenorhabditis elegans	Caenorhabditis elegans	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	High

Sex Applicability

Sex	Evidence
Unspecific	High

Apoptosis is induced in human prostate cancer cell lines (*Homo sapiens*) [Parajuli et al., 2014].

Apoptosis occurs in B6C3F1 mouse (*Mus musculus*) [Elmore, 2007].

Apoptosis occurs in Sprague-Dawley rat (*Rattus norvegicus*) [Elmore, 2007].

Apoptosis occurs in the nematode (*Caenorhabditis elegans*) [Elmore, 2007].

- Apoptosis occurs in breast cancer cells, human and mouse

Key Event Description

Apoptosis, the process of programmed cell death, is characterized by distinct morphology with DNA fragmentation and energy dependency [Elmore, 2007]. Apoptosis, also called “physiological cell death”, is involved in cell turnover, physiological involution, and atrophy of various tissues and organs [Kerr et al., 1972]. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances [Kerr et al., 1972]. Apoptosis is characterized by DNA ladder and chromatin condensation. Several stimuli such as hypoxia, nucleotides deprivation, chemotherapeutical drugs, DNA damage, and mitotic spindle damage induce p53 activation, leading to p21 activation and cell cycle arrest [Pucci et al., 2000]. The SAHA or TSA treatment on neonatal human dermal fibroblasts (NHDFs) for 24 or 72 hrs inhibited proliferation of the NHDF cells [Glaser et al., 2003]. Considering that the acetylation of histone H4 was increased by the treatment of SAHA for 4 hrs, histone deacetylase inhibition may be involved in the inhibition of the cell proliferation [Glaser et al., 2003]. The impaired proliferation was observed in HDAC1$^{-/-}$ ES cells, which was rescued with the reintroduction of HDAC1 [Zupkovitz et al., 2010]. The present AOP focuses on the p21 pathway leading to apoptosis, however, alternative pathways such as NF-kappaB signaling pathways may be involved in the apoptosis of spermatocytes [Wang et al., 2017].

How it is Measured or Detected

Apoptosis is characterized by many morphological and biochemical changes such as homogenous condensation of chromatin to one side or the periphery of the nuclei, membrane blebbing and formation of apoptotic bodies with fragmented nuclei, DNA fragmentation, enzymatic activation of pro-caspases, or phosphatidylserine translocation that can be measured using electron and cytochemical optical microscopy, proteomic and genomic methods, and spectroscopic techniques [Archana et al., 2013; Martinez et al., 2010; Taatjes et al., 2008; Yasuhara et al., 2003].

DNA fragmentation can be quantified with comet assay using electrophoresis, where the tail length, head size, tail intensity, and head intensity of the comet are measured [Yasuhara et al., 2003].

The apoptosis is detected with the expression alteration of procaspases 7 and 3 by Western blotting using antibodies [Parajuli et al., 2014].

The apoptosis is measured with down-regulation of anti-apoptotic gene baculoviral inhibitor of apoptosis protein repeat containing 2 (BIRC2, or cIAP1) [Parajuli et al., 2014].

Apoptotic nucleosomes are detected using Cell Death Detection ELISA kit, which was calculated as absorbance subtraction at 405 nm and 490 nm [Parajuli et al., 2014].

Cleavage of PARP is detected with Western blotting [Parajuli et al., 2014].

Caspase-3 and caspase-9 activity is measured with the enzyme-catalyzed release of p-nitroanilide (pNA) and quantified at 405 nm [Wu et al., 2016].

Apoptosis is measured with Annexin V-FITC probes, and the relative percentage of Annexin V-FITC-positive/PI-negative cells is analyzed by flow cytometry [Wu et al., 2016].

Apoptosis is detected with the Terminal dUTP Nick End-Labeling (TUNEL) method to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [Kressel and Groscurth, 1994].

For the detection of apoptosis, the testes are fixed in neutral buffered formalin and embedded in paraffin. Germ cell death is visualized in testis sections by Terminal dUTP Nick End-Labeling (TUNEL) staining method [Wade et al., 2008]. The incidence of TUNEL-positive cells is expressed as the number of positive cells per tubule examined for one entire testis section per animal [Wade et al., 2008].

- Apoptosis is detected with the Annexin V test

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Event: 2041: Decrease, palatal shelf outgrowth

Short Name: Decrease, outgrowth

Key Event Component

Process	Object	Action
palatal shelves fail to meet at midline	primary palate	decreased
abnormal palatal shelf fusion at midline	secondary palate	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- There are no known differences in palatal outgrowth in terms of sex.
- Life stages- The palate develops early in embryonic development. This begins between the 6^{th} and 12^{th} week of pregnancy in humans and between day 10.0 and 15 in mice (Okuhara and Iseki 2012).
- Taxonomic- Palatal outgrowth is required for proper palate formation in all vertebrates.

Key Event Description

For humans and other mammals, the palate serves as a barrier between the mouth and nasal cavity allowing for simultaneous breathing and eating. The palate consists of an anterior bony hard palate and a posterior muscular soft palate that closes the nasal airways for swallowing and directs airflow to help in generation of speech (Li, Lan et al. 2017). The palate is divided into primary and secondary portions. The primary palate contains the philtrum and the upper incisor region anterior to the incisive foramen while the secondary palate encompasses the remainder of the hard and soft palate (Bush and Jiang 2012). The secondary palate arises during embryonic development as bilateral outgrowths from the maxillary processes. In mammals, these shelves grow first vertically down the tongue before elevating to a position above the dorsum of the tongue where the two shelves meet and fuse to form an intact palate (Ferguson 1988).

How it is Measured or Detected

- Palatal shelf outgrowth can be quantified using imaging techniques such as 3D CT scans during development. Insufficient palatal outgrowth will result in cleft palate. The distance between palatal shelves corelating with outgrowth can be measured and quantified for these individuals.

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List of Adverse Outcomes in this AOP

Event: 2042: Ororofacial clefting

Short Name: OFC

Key Event Component

Process	Object	Action
Cleft palate		increased
cleft upper lip		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:460 - Antagonism of Smoothened receptor leading to orofacial clefting	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

- Sex- OFC can occur for all sexes. Differences in incidence between males and females have been found however a clear understanding of what causes this difference is not understood. Cleft lip with or without cleft palate is more common in males while cleft palate only is more common for females (Barbosa Martelli, Machado et al. 2012).
- Life stages- Orofacial development and any disruption leading to clefting occurs early in embryonic development. This begins between the 6^{th} and 12^{th} week of pregnancy in humans and between day 10.0 and 15 in mice (Okuhara and Iseki 2012).
- Taxonomic- Orofacial development occurs in all vertebrates.

Key Event Description

Orofacial clefts (OFC) are one of the most common birth defects. Orofacial clefts are commonly divided on the anatomy they affect by clefts of the lip and/or palate (CL/P) and those of the palate only (CPO) (Murray 2002). Clefts can also be classified as either syndromic when they occur with other physical or developmental anomalies or nonsydromic in the absence of other symptoms (Stanier and Moore 2004). Like most births, the etiology of OFCs are complex and include a combination of genetic and chemical factors (Lipinski and Bushman 2010, Heyne, Melberg et al. 2015). Orofacial development is tightly regulated by multiple signaling pathways and genes including: fibroblast growth factors (Fgfs), Sonic Hedgehog (shh), bone morphogenic protein (Bmp), transforming growth factor beta (Tgf- β) and transcription factors including Dlx, Pitx, Hox, Gli and T-box (Stanier and Moore 2004). Orofacial development requires precise cell migration, growth, differentiation and apoptosis to create the needed orofacial structures from the oropharyngeal membrane (Jugessur and Murray 2005). During the sixth week of human embryogenesis the medial nasal prominences merge to form the primary palate and the upper lip. The mandibular prominences merge across the midline to produce the lower jaw and lip. Development of the secondary palate begins in the sixth week where the palatal shelves extend internally to the maxillary processes. The shelves then elevate above the tongue and grow towards each other until contact occurs. During weeks 7-8 the

medial edges of the palatal shelves fuse through as series of epithelial-mesenchyme transition (EMT) and apoptosis(Jugessur and Murray 2005, Zhang, Tian et al. 2016). Disruption to the complex processes required for proper orofacial development can occur both through genetic factors and environmental (i.e. chemical) exposure by causing disruption to one or multiple steps of orofacial development resulting in OFC.

How it is Measured or Detected

- OFC can be visually observed both in humans and in animals. It can be classified by which tissues (e.g.cleft lip and palate) are effected and its' severity (complete/incomplete, unilateral/bilateral). Techniques such as the revised Smith-modified Kernahan 'Y' classification can be used describe the type, location, and extent of OFC deformities (Khan, Ullah et al. 2013).

Regulatory Significance of the AO

OFC is one of the most common birth defects occurring in approximately 1 in 700 live births. The etiology of OFC is poorly understood and is believed to be a combination of genetic and environmental factors. Understanding the genetic and environmental factors that can lead to OFC is the first step in preventing this birth defect.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2734: Antagonism Smoothened leads to Decrease, SMO relocation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Low	NCBI

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

The relationship between antagonism of SMO and a decrease in SMO relocation and activation has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects indculding orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

In the absence of SHH ligand, the Patched (PTCH) receptor suppresses the activation of SMO. When HH ligand binds to PTCH, suppression on SMO is released and SMO can relocate, accumulate, and signal to intracellular effectors (Denef, Neubüser et al. 2000, Rohatgi and Scott 2007). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of HH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997). The exact mechanism through which PTCH and SMO interact is not known.

Evidence Supporting this KER

Biological Plausibility

SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade in vertebrates (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009)

Empirical Evidence

- In vitro
  - NIH 3t3 (murine fibroblast) were used to study the effects of three SHH pathway antagonists, SANT 1, SANT2, and cyclopamine on SMO localization using fluorescent microscopy. Cells were treated with increasing concentrations of the antagonists in the presence of SHH ligand. SANT1 and SANT2 both blocked SMO localization in the cilia with IC50 values of 5 and 13nM respectively. Cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10um (>10 fold above kd). All three antagonists inhibited SHH pathway transduction and target gene expression (Rohatgi, Milenkovic et al. 2009).
  - A small molecule screen of 10,000 compounds identified six inhibitors of SHH signaling, four of which bind directly to SMO (SANT1-4). Screening was conducted using NIH 3T3 SHH LightII cells cultured in media conditioned from HEK 293 transfected to stably express Shh-N. Cells were dosed with the compound library at 0.714ug/ml and SHH activity was quantified at 30h using Renilla luciferase activity. A fluorescent binding assay using BODIPY-cyclopamine was used to verify binding to SMO for the SANT compounds. Dose response reported as IC50 for the inhibition of SHH signaling was conducting in NIH 3T3 SHH light2, NIH 3T3 SmoA1-Light2, P2 Ptch1-/- (mouse embryonic fibroblasts) (Chen, Taipale et al. 2002).

Compound/Cell	SHH-Light2 (nM)	SmoA1-Light2 (nM)	Ptch1-/- (nM)
SANT-1	20	30	20
SANT-2	30	70	50
SANT-3	100	80	80
SANT-4	200	300	300

- Direct binding of cyclopamine to SMO was verified using a photoaffinity form of cyclopamine (PA-cyclopamine). PA-cyclopamine had previously been shown to inhibit SHH signaling in NIH 3T3 Shh-LightII cells with similar IC50 values to cyclopamine (300nm and 150nm respectively) (Taipale, Chen et al. 2000). Binding to SMO was verified using a COS-1 (fibroblast, monkey) line transfected to over express SMO. The location of cyclopamine binding was further investigated using BODIPY- cyclopamine and COS-1 cells modified to lack either a N-terminal, extracellular cysteine-rich domain, or the cytoplasmic C terminal of SMO. The findings support that cyclopamine does not require these domains and instead binds directly to the heptahelical domain (Chen, Taipale et al. 2002).
- To investigate whether SMO localization is regulated by SHH, a renal epithelial MDCK (Madin-Darby canine kidney) line was engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit, Aanstad et al. 2005)
- To determine whether PTCH1 regulates localization of SMO MEFs from $PTCH1^{-/-}$ mice were used. These showed SHH activity and SMO localization in the primary cilium in the absence of SHH ligand or SAG. Reintroduction of PTCH1 via a retrovirus suppressed SHH activity and prevented SMO accumulation in primary cilia (Rohatgi and Scott 2007)
- A high content assay to detect compounds that block SMO accumulation to the primary cilia in the presence of SHH was used to screen a library of ~5600 compounds. This screen identified 26 hits with DY131 and its analog GSK4716 further investigated as potent hits. These compounds inhibited SHH induced accumulation of SMO::EGFP with IC50s of 0.8um and 2um respectively. DY131 and GSK4716 both inhibited the activation of a Glireporter with IC50s of 2um and 10um respectively (Wang, Arvanites et al. 2012).

- In vivo
  - The presence of critical periods for disruption of SHH was investigated using C57BL/6J mice. Vismodegib was suspended at 3mg/ml in 0.5% methyl cellulose and 0.2% tween. Pregnant dams were administered 40mg/kg vismodegib at GD7.0, 7.25, 7.5, 7.75, 8.0, 8.25,8.5, 8.625, 8.75, 8.875, 9.0, 9.25, 9.5, 9.75, and 10.0. Cyclopamine was dosed at 120mg/kg/d via subcutaneous infusion between GD8.25-9.375. Pregnant dams were euthanized at GD17 and fetal specimens were collected and fixed for imaging. The control group consisted of fetuses exposed to 0.5% methyl cellulose and 0.2% tween at GD7.75, 8.875, or 9.5. Acute exposure to vismodegib resulted in a peak incidence of lateral cleft lip and palate at GD8.875 (13%). Exposure at GD9.0 and 10.0 resulted in clefts of the secondary palate only (34%). A higher penetrance (81%) was found for cyclopamine exposure (Heyne, Melberg et al. 2015).
  - Two-week-old mice were dosed with 40mg/kg vismodegib (GDC-0449) via ip injection twice a day for 3 consecutive days. Quantification of immunofluorescence and ciliary length showed that like $SMO^{fl/+}$ mice, ciliary M71/M72 OR was reduced while cilia lengths were not changed. To determine if SMO regulates ciliary localization an OMP-CRE mouse line was used. It was found that immunofluorescence of M71/M72 was reduced in both $SMO^{fl/+}$, $SMO^{fl/fl}$, as compared to $SMO^{+/+}$ control (Maurya, Bohm et al. 2017).
  - To explore how a conditional loss of primary cilia on neural crest cells $Kif3a^{f/f}$ Wnt1-Cre mice were used to explore the molecular basis of aglossia. Aglossia was found to be due to a lack of mesoderm derived muscle precursor migration. RNA-seq was used on E11.5 embryos on the mandibular prominces of wildtype and knock mice. The key SHH readout, GLI1 was downregulated two-fold in mutants (Millington, Elliott et al. 2017).
  - Cyclopamine was found to inhibit SHH signaling in White leghorn neural plate explants. Explants were dissected from stage 9-10 embryo chicks and cultured in collagen gels. Tissues were cultured in Shh-N media from COS-1 cells. Cyclopamine was dissolved in ethanol and added to test tissues. Tissues were fixed at 24-29hr and processed for immunofluorescence. 120nm cyclopamine was found to repress SHH induction as determined by Pax7 repression and the blockage of floor plate and motor neuron induction (Incardona, Gaffield et al. 1998).
    - Multiple ciliopathies associated with clefting in humans including Meckel-Gruber syndrome (OMIM 249000) and Ellis-van Creveld syndrome (OMIM 225500)(Brugmann, Cordero et al. 2010)

Uncertainties and Inconsistencies

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

An endogenous ligand for SMO has not been discovered although evidence for one exists and that PTCH controls SMO by controlling its' availability or accessibility. To support this, it has been shown that PTCH and SMO do not physically interact (Chen and Struhl 1998). PTCH acts catalytically with SMO with one PTCH receptor capable of controlling many (~50) SMO receptors (Taipale, Cooper et al. 2002). Since PTCH includes a sterol sensing domain and shares characteristics of ancient bacterial transporters, a model of PTCH functioning by pumping a sterol-like MSO regulator has been proposed (Mukhopadhyay and Rohatgi 2014). SMO is constitutively active in the absence of PTCH suggesting that the elusive molecule is an agonist (Rohatgi and Scott 2007). Conversely, the discovery that oxysterols bind to the CRD binding domain acting as positive modulators suggest that the molecule could be an agonist with PTCH functioning to sequester away or limit cellular concentration (Corcoran and Scott 2006, Nachtergaele, Mydock et al. 2012)

The activity of SMO is controlled by ligand binding (Kobilka 2007). Two separate binding pockets, one in the groove of the extracellular CRD and the other in the helices of the TMD have been identified (Nachtergaele, Mydock et al. 2012, Rana, Carroll et al. 2013, Wang, Wu et al. 2013, Byrne, Sircar et al. 2016, Huang, Zheng et al. 2018). These two binding pockets have been shown to interact in an allosteric manner (Nachtergaele, Mydock et al. 2012). The binding pocket in the helices of the TMD binds several SMO agonists including SAG as well as antagonists Vismodegib and Sonidegib. The CRD binding pocket binds cholesterol and its' oxidized derivates (Byrne, Luchetti et al. 2018). The antagonist cyclopamine binds to the TMD binding pocket and inhibits SHH signal transduction. However, in mSMO carrying the mutations D477G/E552K that disable the TMD binding pocket, cyclopamine binds to the CRD pocket and activates the pathway (Huang, Nedelcu et al. 2016). To date several oxysterols including 20(S)-hydroxylcholesterol, 22(S)-hydroxylcholesterol, 7-keto-25-hydroxylcholesterol and 7-keto-27-hydroxylcholesterol have been identified as activators of SMO (Dwyer, Sever et al. 2007, Nachtergaele, Mydock et al. 2012, Myers, Sever et al. 2013). A binding site for 24(S),25-epoxycholesterol has been identified in the TMD pocket using cryo-EM of SMO in complex with 24(S),25-epoxycholesterol (Qi, Liu et al. 2019).

While it is well understood that cyclopamine is an antagonist of SMO, contradictory in vivo data was found regarding whether cyclopamine blocks SMO relocation to the primary cilia. Rohatgi et al used NIH 3T3s cell and found that cyclopamine did not inhibit the accumulation of SMO in the cilia even when dosed at 5-10um (>10 fold above kd). All three antagonists inhibited SHH pathway transduction and target gene expression (Rohatgi, Milenkovic et al. 2009). Corbit et al used a renal epithelial MDCK (Madin-Darby canine kidney) line was engineered to express Myc-tagged SMO. Following culture for 1hr in SHH conditioned media SMO presence in the primary cilium is upregulated while cells cultured in the presence of cyclopamine see a downregulation of SMO in the primary cilia (Corbit, Aanstad et al. 2005). Further work is required to determine if SMO antagonism via cyclopamine results in decrease in SMO relocation.

Quantitative Understanding of the Linkage

Time-scale

Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016). No data was found on how fast antagonism of SMO will stop its' relocation to the primary cilia.

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Relationship: 2735: Decrease, SMO relocation leads to Decrease, GLI1/2 translocation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

The relationship between a decrease in translocation of SMO and a decrease in GLI1/2 translocation to the nucleus has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Smoothened (SMO) receptor is Class F G protein coupled receptor involved in signal transduction of the Sonic Hedgehog (SHH) pathway. It includes distinct functional groups including ligand binding pockets, cysteine rich domain (CRD), transmembrane helix (TM), extracellular loop (ECL), intracellular loop (ICL), and a carboxyl-terminal tail (C-term tail) (Arensdorf, Marada et al. 2016). SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016).

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated, the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009). Multiple ciliopathies are associated with clefting in humans including Meckel-Gruber syndrome (OMIM 249000) and Ellis-van Creveld syndrome (OMIM 225500)(Brugmann, Cordero et al. 2010).

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to an increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016). Downstream transcription is primarily activated by Gli2 and repressed by Gli3 (Wang, Fallon et al. 2000, Bai, Auerbach et al. 2002, Persson, Stamataki et al. 2002). Gli1 serves primarily as an activator of transcription and works through amplification of the activated state (Park, Bai et al. 2000).

Evidence Supporting this KER

Biological Plausibility

SMO signaling is dependent upon its relocation to a subcellular location. This relocation occurs in the primary cilium (PC) in vertebrates (Huangfu and Anderson 2005). It has been shown that SMO localization to the tip of the primary cilia is essential for the SHH signaling cascade via the GLI transcription factors(Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Rohatgi, Milenkovic et al. 2009)

Empirical Evidence

- In vitro
  - NIH 3T3 clones with stable HA-Gli2 expression were created and a line with low HA-Gli2 expression was selected for further study. The reporter activity was induced by ShhN and fully inhibited by cyclopamine. When stimulated with ShhN, antibody staining was used to verify that Gli2 accumulates at the tip of the primary cilia. Immunostaining was also used to find that Gli2 accumulated in the nucleus of cells treated with ShhN. Using nuclear extracts of unstimulated cells HA-Gli2R was predominantly localized in the nucleus while in stimulated cells HA-Gli2 increased and HA-Gli2 decreased. Cells treated with Shh agonist SAG also had SMO accumulation in the primary cilia and increased HA-Gli2A in the nucleus (Kim, Kato et al. 2009).
  - NIH 3T3 cells were used to study whether the oxysterols and/or cholesterol are required for SHH signaling. Cells were depleted of sterols via incubation with methyl-β-cyclodextrin (MCD). Fluorinated sterols were added back as soluble components and the cells were stimulated with Shh ligand. Assays were performed for recruitment of endogenous SMO to the primary cilia and for pathway activation using a transcriptional reporter assay. Sterol depletion blocked relocation of SMO to the cilia and SHH activation. Cholesterol and 25-fluorocholesterol both rescued sterol depleted cells and restored SHH pathway activation (Huang, Nedelcu et al. 2016).
  - MMS1 (human myeloma) cells were used to study whether activation of Gli1 is required for its' translocation to the nucleus. Forskolin (FSK) which acts by blocking GLI1 access to PKA was added to culture for 24h at 10µm. The nuclear localization of GLI1 was significantly decreased in the Prescence of FSK (Blotta, Jakubikova et al. 2012).
- In vivo
  - To explore how a conditional loss of primary cilia on neural crest cells, Kif3a$^{f/f}$ Wnt1-Cre mice were used to explore the molecular basis of aglossia (congenital absence of tongue). Aglossia was found to be due to a lack of mesoderm derived muscle precursor migration. RNA-seq was used on E11.5 embryos on the mandibular prominces of wildtype and knock mice. The key SHH readout, GLI1 was downregulated two-fold in mutants (Millington, Elliott et al. 2017).
  - To test whether vertebrate SUFU, in situ hybridization was used to analyze SUFU expression in mouse embryos at 8.5-15.5 days post-coitum. The expression was found to partially overlap with expression of PTCH and GLI1-3 supporting a role of SUFU in SHH signaling. Radioactive in situ hybridization was used to analyze expression of SUFUH and PTCH1 in a 12-week-old human embryo. SUFUH expression was to be preferentially expressed in cells that receive SHH signaling (Kogerman, Grimm et al. 1999)
  - Multiple ciliopathies associated with clefting in humans including Meckel-Gruber syndrome (OMIM 249000) and Ellis-van Creveld syndrome (OMIM 225500)(Brugmann, Cordero et al. 2010).

Uncertainties and Inconsistencies

While we know that entry to the cilia is tightly controlled, the exact mechanism of SMO ciliary trafficking is not fully understood. The PC is separated from the plasma membrane by the ciliary pockets and the transition zone which function together to regulate the movement of lipids and proteins in and out of the organelle (Goetz, Ocbina et al. 2009, Rohatgi and Snell 2010). The SHH receptor PTCH contains a ciliary localization sequence in its' carboxy tail. Localization of PTCH to the PC is essential for inhibition of SMO as deletion of the CLS in PTCH prevents PTCH localization as well as inhibition of SMO (Kim, Hsia et al. 2015) (53). SMO also contains a CLS, but only accumulates in the PC upon ligand binding (Corbit, Aanstad et al. 2005). The entry of SMO into the PC is thought to occur either laterally through the ciliary pockets or internally via recycling endosomes (Milenkovic, Scott et al. 2009). Once inside the PC, SMO can diffuse freely, however it will usually accumulate in specific locations depending upon its' activation state. Inactive SMO will accumulate more at the base of the PC while active SMO will accumulate in the tip of the PC (Milenkovic, Weiss et al. 2015).

Quantitative Understanding of the Linkage

The data presented in support of this KER includes both in vitro and in vivo studies. The in vitro work offers data that SMO relocates to the tip of the primary cilium and that this plays a role in the translocation of the GLI transcription factors to the nucleus. The in vivo work shows that loss of cilia in embryonic mice leads to a significant decrease in GLI1 in the nucleus. SUFU expression was found to overlap with PTCH1 expression in both mice and human embryos supporting that SUFU plays a role in SHH signal transduction.

Time-scale

Relocation of SMO to the PC typically occurs within ~20 minutes of agonist stimulation (Arensdorf, Marada et al. 2016). No data was found with regards to GLI1/2 translocation.

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Relationship: 2721: Decrease, GLI1/2 translocation leads to Decrease, GLI1/2 target gene expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	

All presented evidence for the relationship is performed in mice. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

The Glioma-associated onocogene (Gli) family of zinc finger transcription factors (Gli1, Gli2, Gli3) are the primarily downstream effectors of the Hedgehog (HH) signaling cascade. When HH ligand binds to Patched (PTCH), its' inhibition on SMO is relieved. SMO this then able to accumulate to the tip of primary cilium in its' active form (Corbit, Aanstad et al. 2005, Rohatgi, Milenkovic et al. 2007, Kim, Kato et al. 2009). SMO causes the GLI family to become dislodged from their complex with the negative regulator of HH signaling, Suppressor of Fused (Sufu) (Kogerman, Grimm et al. 1999, Pearse, Collier et al. 1999, Stone, Murone et al. 1999, Tukachinsky, Lopez et al. 2010). The GLI-Sufu complex maintains retention of Gli in the cytosol allowing for exposure to phosphorylation via protein kinase A (PKA) which inhibits downstream signal transduction (Tuson, He et al. 2011). When SMO is activated, the GLI2/3-Sufu complex is dismantled allowing for retrograde transport of GLI back into the nucleus (Kim, Kato et al. 2009). This relocation then leads to signaling to effectors resulting in the activation of the GLI transcription factors and the subsequent induction of SHH target gene expression (Alexandre, Jacinto et al. 1996, Von Ohlen and Hooper 1997).

The GLI family is found in both a long activator form (GliA) or a proteolytically cleaved repressor form (GliR). Current understanding is that Gli3 functions primarily as a repressor while Gli1 and Gli2 function mainly as activators of the pathway and that recruitment of SMO to the cilium leads to an increase in the ratio of GliA:GliR (Hui and Angers 2011, Liu 2016). Downstream transcription is primarily activated by Gli2 and repressed by Gli3 (Wang, Fallon et al. 2000, Bai, Auerbach et al. 2002, Persson, Stamataki et al. 2002). Gli1 serves primarily as an activator of transcription and works through amplification of the activated state (Park, Bai et al. 2000).

Evidence Supporting this KER

The evidence presented for this KER is low.The relationship between GLI1/2 translocation and a decrease in GLI1/2 target gene expression relocation has been shown indirectly in multiple mouse models through disruption of SHH signaling at the level of SMO. From our understanding of the SHH pathway, we can infer that disruption of the SHH signaling pathway at the level of SMO is causing a decrease in GLI1/2 translocation and it is this that is causing the altered gene expression While clear evidence that disruption of SHH signaling leads to altered gene expression especially those of the Fox family, insufficient evidence exists for the direct relationship between GLI1/2 translocation and SHH target gene expression. The evidence also lacks direct human applicability as all presented work was performed *in vitro* on murine models or *in vitro* on murine cell lines.

Biological Plausibility

SHH signaling is well established to be essential for proper embryonic development in vertebrates including mice and humans.

Activation of the pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018).

Empirical Evidence

- In vitro
  - A mouse cNCC line (09-1) with the expression signature (AP-2alpha (Tfap2a, Twist1, Sox9, Cd44) was used to study whether foxf2 is a target of SHH signalling. Addition of SHH ligand (0.4µg/ml) was found to upregulate both GLI1 and Foxf2. This upregulation was completely blocked by the addition of vismodegib (120nm)(Everson, Fink et al. 2017).
  - To determine if SHH pathway inhibition was downstream for GANT 61 and GANT 58, a Sufu-null MEF cell line was used. Treatment of cells with either GANT at 10µm led to a significant reduction of SHH target genes GLI1 and Hip1 as determined by qPCR. As expected, cyclopamine was unable to inhibit signalling in this system as activation occurs downstream of SMO. GANT 61 is believed to act through addition of the modification to GLI1 that compromises its' ability to properly bind DNA (Lauth, Bergström et al. 2007).
  - GLI activators bind to the GACCACCCA motif to promote transcription of GLI1, PTCH1, PTCH2, HHIP1, MYCN, CCND1, CCND2, BCL2, CFLAR, FOXF1, FOXL1, PRDM1 (BLIMP1), JAG2, GREM1, and Follistatin (Katoh and Katoh 2009)
- In vivo
  - In situ hybridization was used to determine expression of GLI1 in C57BL/6J mice to better understanding temporal SHH signalling. At GD 9.0 no difference was found between control and embryos exposed to cyclopamine (120mg/kg/day). GLI1 was downregulated in the ventral frontonasal prominence (FNP) of clomipramine exposed embryos by GD 9.25. FNP tissue was micro dissected and cDNA microarray analysis was performed. 210 genes were found to be dysregulated including a significant enrichment to the forkhead box (Fox) family. RT-PCR confirmed significant down regulation of the SHH target genes GLI1 and PTCH1 as well as nine Fox members: Foxa2, Foxb2, Foxc1, Foxc2, Foxd1, Foxe1, Foxf1, Foxf2, Foxl1. Two members of the fox family, Foxm1 and Foxo1 were not found to differentially expressed in either the cDNA microarray or RT-PCR (Everson, Fink et al. 2017).
  - Using mutant Osr2-IresCre;$Smo^{c/c}$ mice Foxf2 and Foxf1were found to be positively regulated by SHH-SMO signalling. Expression of Osr2 was found to be reduced by E13.5 in the mutants. Expression of Osr1, Pax9, Tbx22 were not found to be altered (Lan and Jiang 2009).
  - To study whether SHH signalling regulates the developmental fate of the ecto-mesenchyme via regulation of gene activity in the facial primoridia, Wnt1-Cre;$Smo^{n/c}$, (removal of SHH signalling) and Wnt1-Cre;R26SmoM2 (activation of SHH signalling). Positive regulation from SHH activity was found for Foxc2, Foxd1, Foxd2, Foxf1, and Foxf2. The Fox genes were found to be dissimilar in expression pattern with spatial activation even with uniform activation of the SHH pathway. Foxc2 and Foxd1 were found to be expressed ubiquitously in the MNA except at the midline, while Foxf1 is expressed at the lateral ends. Foxd2 and Foxf2 are both expressed along the mediolateral axis with Foxd2 having an increasing gradient from medial to lateral and Foxf2 having an opposing gradient (Jeong, Mao et al. 2004).

Quantitative Understanding of the Linkage

The quantitative understanding for this KER is low. Studies to investigate response-response relationship as well as time scale have not been conducted or were not found in the literature review. The empirical evidence presented establishes that disruption of SHH signaling results in the altered gene expression of SHH target genes.

Known Feedforward/Feedback loops influencing this KER

Positive feedback loop of gene expression from GLI1 and negative feedback loop for PTCH1, PTCH2, HHIP1 (Katoh and Katoh 2009)

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Relationship: 2731: Decrease, GLI1/2 target gene expression leads to Decrease, SHH second messenger production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence

Sex	Evidence
Unspecific	High

The relationship between a decrease in shh target gene expression and a decrease in secondary messenger production has been shown in mouse models. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

A network of reciprocal growth factor signaling between the epithelium and mesenchyme is required for proper growth and patterning of the early palatal shelves. This signaling is largely comprised of a network between bone morphogenic protein (BMP), Fibroblast growth factor (Fgf), and Sonic Hedgehog (SHH) (Zhang, Song et al. 2002, Rice, Spencer-Dene et al. 2004). Activation of the SHH pathway results in a downstream signaling cascade resulting in the relocation of GLI to the nucleus and subsequent gene transcription (Carballo, Honorato et al. 2018). This gene expression drives secondary messenger signaling for the pathway. Proper Msx1 activity in the mesenchyme is required for the expression of SHH in the overlying epithelium (Zhang, Song et al. 2002). Maintenance of SHH expression in the epithelium is believed to be dependent on Fgf10 expression in the mesenchyme and its' signaling through Fgfr2b in the epithelium (Rice, Spencer-Dene et al. 2004).

Evidence Supporting this KER

Empirical Evidence

- In Osr2-IresCre;Smo$^{c/c}$ (SHH pathway inactive) mutant mice Fgf10 mRNA was found to be significantly reduced in the anterior palatal mesenchyme. The expression of Fgf10 correlated with a downregulation of PTCH1 (Lan and Jiang 2009).
- To determine if SHH can induce Fgf10, SHH overexpressing cells were implanted in the anterior region of the wing bud of chick embryos. By 27 hours, the expression of Fgf10 had significantly increased and expanded from the anterior mesenchyme to the bifurcating wing bud (Ohuchi, Nakagawa et al. 1997).
- To investigate whether MSX-1 is in the same pathway as Fgf10, MSX-1 expression was examined in Fgf10-/- mice and Fgf10 expression was examined in Msx-1-/- mice. No change in expression was found and it is concluded that MSX-1 is not a downstream target of Fgf10 (Alappat, Zhang et al. 2005).
- SHH expression is reduced in the palatal epithelium of both Fgf10-/- and Fgfr2b -/- mutants. Exogenous Fgf10 induced SHH in WT palatal epithelium (Rice, Spencer-Dene et al. 2004).
- BMP2 and BMP4 is downregulated in the anterior palate of Osr2-IresCre;Smo$^{c/c}$ (SHH pathway inactive) mutant mice (Lan and Jiang 2009).
- Upregulation of mesenchymal BMP4 by SHH via Foxf1 or Foxl1 (Katoh and Katoh 2009).

Uncertainties and Inconsistencies

The relationships and feedback/feedforward loops that exist between SHH and its' secondary messengers primary Fgf10 and BMP4 is not well understood. Some evidence exists that expression of both Fgf10 and BMP4 correlates with that of SHH. The state of evidence is lacking and no dose response data was found.

Relationship: 2722: Decrease, GLI1/2 target gene expression leads to Decrease, Cell proliferation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent	Low	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	Low	NCBI

Life Stage Applicability

Life Stage	Evidence

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	High

The relationship between a decrease SHH second messengers and a decrease in proliferation has been shown repeatedly in mice models as detailed in the empirical evidence section. The relationship is biologically plausible in human, but to date no specific experiments have addressed this question. The SHH pathway is well understood to be fundamental to proper embryonic development and that aberrant SHH signaling during embryonic development can cause birth defects including orofacial clefts (OFCs). For this reason, this KER is applicable to the embryonic stage with a high level of confidence.

Key Event Relationship Description

SHH is well understood to regulate cell proliferation during development. Shh regulation of proliferation works at least in part through regulation of cyclin D1 (Ccnd 1) and Ccnd 2 (Kenney and Rowitch 2000, Ishibashi and McMahon 2002, Lobjois, Benazeraf et al. 2004, Mill, Mo et al. 2005).

Evidence Supporting this KER

Empirical Evidence

- In vivo
  - In mouse palate explants application of SHH was found to induce proliferation in the palatal mesenchyme as measured by BrdU (Rice, Spencer-Dene et al. 2004).
  - In CD-1 WT and MSX-1-/-, SHH soaked beads were able to induce proliferation in palatal mesenchyme explants at 24hr but not after 8hr suggesting the induction of proliferation is through an indirect mechanism (Zhang, Song et al. 2002).
  - IHC staining for Ccnd-1 and Ccnd-2 in Osr2-IresCre $Smo^{c/c}$ (SHH inactive) and control embryos was used to determine if expression patterns differed between the mesenchyme and epithelium in mutants. Expression for both Ccnd-1 and Ccnd-2 was found to be reduced in the mesenchyme for mutants. mRNA was found to be reduced for both Ccnd-1 and Ccnd-2 in the palatal mesenchyme (Lan and Jiang 2009).

Uncertainties and Inconsistencies

The regulation of proliferation by shh and its' regulation of ccnd-1 and ccnd-2 has not been well studied. The relationship is biologically plausible and the studies presented suggest that some of the regulation of proliferation is due to gene expression of ccnd-1, ccnd-2. Further studies are needed to further out understanding of the regulation of proliferation by shh.

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Relationship: 2724: Decrease, Cell proliferation leads to Decrease, outgrowth

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent		

Relationship: 2726: Decrease, outgrowth leads to OFC

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonism of Smoothened receptor leading to orofacial clefting	adjacent		