

THE BITTER TASTE RECEPTOR TAS2R14 IS EXPRESSED IN OVARIAN  
CANCER AND MEDIATES APOPTOTIC SIGNALLING

by

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## DEDICATION PAGE

To my grandparents, Christina, Frank, Brenda and Bernie, and my parents, Angela and Tom – for teaching me the value of hard work.

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## **ABSTRACT**

Bitter taste receptors (Tas2Rs) are a subfamily of G-protein coupled receptors expressed not only in the oral cavity but also in several extra-oral tissues and disease states. For example, mammary epithelial cells express Tas2Rs, and their expression is downregulated in breast cancer. Tas2R expression was also observed in colon and pancreatic cancer, among others. As several natural bitter compounds from plants have displayed beneficial effects in ovarian cancers, the expression of several Tas2R subtypes was characterized in ovarian cell lines and patient tissue samples and their functionality was determined. Our qPCR analysis of 5 *TAS2Rs* shows that mRNA expression of several Tas2Rs was significantly reduced in ovarian cancer cells when compared to healthy tissue. Tas2R14 was also shown to be expressed using immunohistochemistry and immunocytochemistry on epithelial ovarian carcinoma tissues. Tas2R proteins were also expressed in various ovarian cancer cell lines and their expression was decreased with receptor-specific siRNAs. Noscipine stimulation of ovarian cancer cells resulted in an effect on apoptosis that was receptor-dependent. Our results demonstrate that Tas2Rs are expressed in ovarian cancer and their activation has an impact on cell survival.

## LIST OF ABBREVIATIONS AND SYMBOLS USED

7TM	7 transmembrane-spanning
AV	annexin-V stain
AC	adenylate cyclase
Bcl-XL	B-cell lymphoma-extra large
Ca <sup>2+</sup>	calcium ion
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
cNMP	cyclic nucleoside monophosphate
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ENaC	epithelial sodium channel (salt taste receptor)
EOC	epithelial ovarian cancer
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FN	normal fallopian tube tissue
G $\alpha$	G alpha subunit
G $\alpha_{\text{gust}}$	gustducin
G $\beta$	G beta subunit
G $\gamma$	G gamma subunit
GAP	GTPase activating protein
GCRDb	G-protein Coupled Receptor Database (now defunct)
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GFP	green fluorescence protein
GLP-1	glucagon-like peptide 1
GPCR	G-protein coupled receptor
GPCRDB	GPCR Database (currently in use)
GRAFS	GPCR classification system in humans (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin)
GTP	guanosine triphosphate
HEK-293A	human embryonic kidney cells 293, human adenovirus type 5 transformed
HGSEOC	high grade serous epithelial ovarian cancer
hTas2R	human bitter taste receptor
ICC	immunocytochemistry
IHC	immunohistochemistry
IP <sub>3</sub>	inositol 1,4,5-triphosphate
IUPAC	International Union of Pure Applied Chemistry
K <sup>+</sup>	potassium ion
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
mRNA	messenger RNA
mTas2r	mouse bitter taste receptor

Na <sup>+</sup>	sodium ion
ON	normal ovarian tissue
ORF	open reading frame
OT	ovarian tumour tissue
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIP <sub>2</sub>	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PLD	phospholipase D
PROP	6-n-propyl-2-thiouracil
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
Rac-1	Ras-related C3 botulinum toxin substrate 1
SCC	solitary chemosensory cells
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
TAS1R	Taste receptor type 1 – sweet/umami taste receptor (gene or mRNA)
Tas1R	Taste receptor type 1 – sweet/umami taste receptor (protein)
TAS2R	Taste receptor type 2 – bitter taste receptor (gene or mRNA)
Tas2R	Taste receptor type 2 – bitter taste receptor (protein)
TRC	taste receptor cell
TRPM5	transient receptor potential cation channel protein M family member 5
UN	normal uterine tissue

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# CHAPTER 1 INTRODUCTION

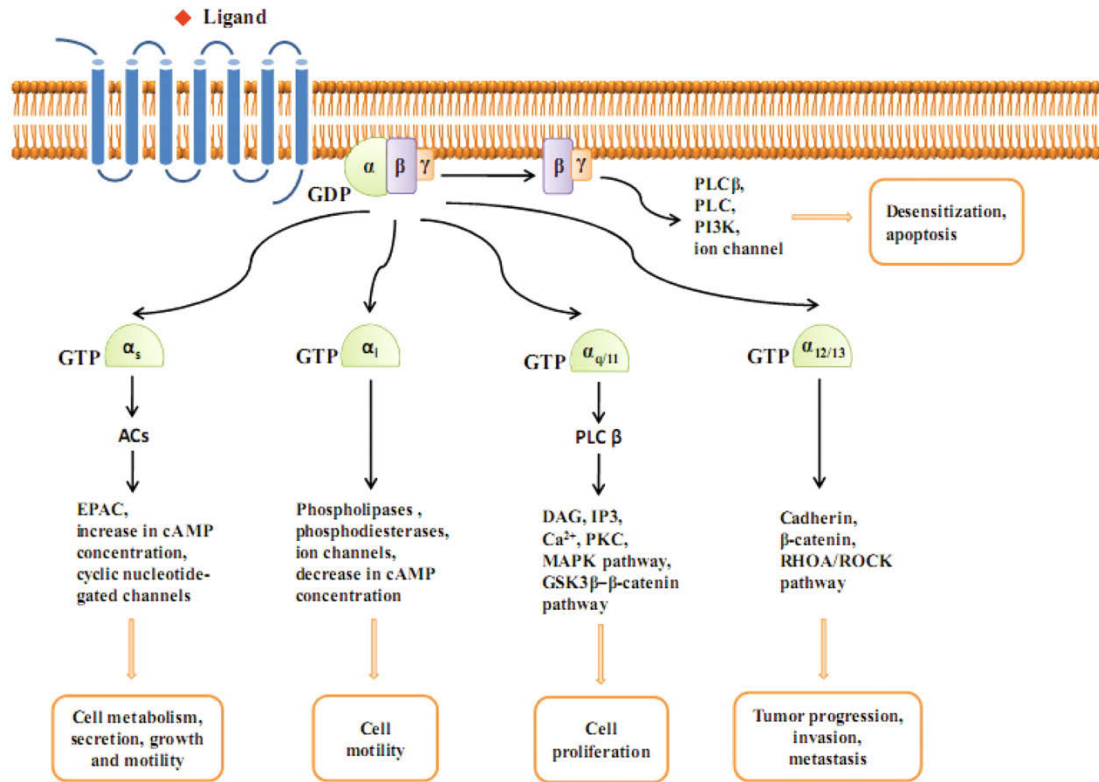
## 1.1 G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors (GPCRs) comprise the largest family of cell surface signaling receptors and one of the largest protein superfamilies encoded in the human genome, and mediate many neurological, physiological and environmentally activated responses<sup>1-3</sup>. The expansive GPCR superfamily is not only large in number, but exceptionally diverse in signal recognition. Nearly half of all GPCR genetic sequences in humans encode odorant and pheromone receptors, which are responsible for perceiving scent molecules<sup>4</sup>; however, GPCRs may also recognize photons, hormones, neurotransmitters and small molecules among others<sup>5</sup>. Presently, GPCR drugs comprise an estimated 30% of all pharmaceuticals on the market<sup>6</sup> and two out of the top ten selling drugs in 2015, aripiprazole and fluticasone-salmeterol, act on GPCRs<sup>7</sup>. Given that GPCRs are present on cells of nearly every organ system, GPCRs are thus of interest in the development of novel drug therapies<sup>8</sup>.

GPCRs are evolutionarily conserved across the tree of eukaryotes<sup>9, 10</sup>. Indeed, analyses of the genomes of early eukaryotic species such as the diatom *Thalassiosira pseudonana* and the placozoan *Trichoplax adhaerens* uncovered vertebrate-like GPCR repertoires with 99.4-99.8% sequence homology to those encoded in the human genome<sup>11</sup>. This high level of sequence identity suggests that GPCRs changed little since their emergence in

eukaryotic genomes, highlighting once more the overall importance of GPCRs in cellular signaling.

GPCR activity is generally explained through the canonical model of GPCR signaling. In this model, inactive GPCRs are associated with a heterotrimeric  $G\alpha\beta\gamma$  protein complex on the cytoplasmic side of the membrane composed of alpha ( $\alpha$ ), beta ( $\beta$ ) and gamma ( $\gamma$ ) subunits bound to low energy guanosine diphosphate (GDP, Figure 1). Upon binding an agonist molecule, the GPCR is activated and, acting as a guanine nucleotide exchange factor (GEF), exchanges a GDP molecule associated with the  $G\alpha$  subunit for a GTP molecule<sup>12</sup>. Once bound to GTP, the now active  $G\alpha$  and  $G\beta\gamma$  subunits functionally and/or physically dissociate and go on to regulate effector molecules such as adenylyl cyclase, phospholipases C and D, phosphoinositide 3-kinase (PI3K) and intracellular calcium<sup>12</sup>. These effector molecules have many targets, and depending on which  $G\alpha$  subtype is activated through GPCR ligand binding, can positively regulate (activate) or negatively regulate (inactivate) many downstream signaling cascades involved in cellular communication, growth, survival and cell death<sup>13</sup>. Thus, a thorough understanding of GPCR signaling in a given tissue or disease can further the development of targeted pharmaceutical strategies.



**Figure 1** **GPCR-mediated signaling pathways.** Upon activation by extracellular ligands, GPCRs can regulate key biological functions, such as cell proliferation, metabolism, secretion, motility, tumour progression, invasion, and metastasis, through signaling pathways mediated by the four G protein  $\alpha$  subunits ( $G_s$ ,  $G_i$ ,  $G_{q/11}$ , and  $G_{12/13}$ ). Adapted with permission from Humana Press – Cancer Genomics and Proteomics: Methods and Protocols (Wu et al., 2012)<sup>14</sup>, Copyright © 2017.

## 1.2 GPCR CLASSES

GPCRs, as mentioned previously, are highly similar across eukaryotic species<sup>11</sup>. Despite this, there exists a high level of structural and biochemical variability among the GPCR superfamily, and it is due to this variation that we observe how these 7 transmembrane-spanning (7TM) cell surface receptors can regulate highly diverse signaling cascades. Developing classification systems to encompass all GPCRs has thus been difficult, and both previous and current systems have relied upon comparison of amino acid sequences, structural features, ligand recognition motifs and physiological signaling partners<sup>15, 16</sup>. Attwood and Findlay were the first to introduce a classification system in 1993 by developing a sequence-based “fingerprint” technique aimed at characterizing the 7TM hydrophobic domains of GPCRs and sorting them into several “clans” based on their sequence homology<sup>17, 18</sup>. The classification system commonly used today can trace its roots to Kolakowski’s seven membrane classification system, created one year following Attwood and Findlay’s, based on sequence homology<sup>19</sup>. In Kolakowski’s now defunct database GCRDb, all G-protein binding receptors were classified through an A-F classification, with an additional class, O, reserved for 7TM proteins that do not associate with G-proteins.

Currently, a six-class version (A-F) of the Kolakowski system is used to classify GPCRs. This six class version is useful in that it encompasses all eukaryotic GPCRs; however, some classes such as the Class D fungal pheromone receptors and the Class E cAMP receptors (that to-date have been found only in lower eukaryotic species) are irrelevant in

the study of human specific GPCRs. Thus, the GRAFS classification system (an acronym for Glutamate, Rhodopsin, Adhesion, Frizzled/Tas2 and Secretin) was developed to more accurately represent the signaling systems encoded in the human genome<sup>2</sup>.

The rhodopsin-like receptors are the largest class of GPCRs encompassing some ~ 670 receptors, and includes all opioid, cannabinoid, dopamine and olfactory receptors<sup>20</sup>. Most rhodopsin-like receptors have short N-termini without many conserved domains, and bind a large variety of ligands such as peptides and amines. Rhodopsin-like receptors are also the class of GPCRs that are the most highly targeted in clinical applications<sup>21</sup>. Secretin-like receptors are smaller in number and include hormone receptors such as those that bind calcitonin, parathyroid hormone, glucagon and their namesake secretin<sup>22</sup>, while those of the glutamate receptor family include GPCRs involved in neurotransmission such as metabotropic glutamate receptors, sweet and umami taste receptors (Tas1R1-3) and the calcium sensing receptor (CASR)<sup>21, 23</sup>. Finally, the frizzled/Tas2R receptors includes those receptors that are important for embryonic development and taste perception<sup>12</sup>. To-date, there have been no therapeutics designed and approved that target frizzled receptors. This figure may well soon change as several of these receptors have been implicated in tumourigenesis<sup>24, 25</sup>, indicating their potential as targets for the development of novel chemotherapeutics.

### 1.3 GPCR SIGNALING THROUGH G PROTEINS

Human G proteins are divided into four families based on sequence similarity and signaling output:  $G_s$ ,  $G_q$ ,  $G_{12/13}$  and  $G_{i/o}$ <sup>26</sup>.  $G_s$  proteins initiate a stimulatory cascade, and their activity ultimately leads to the activation of adenylyl cyclase (AC), an enzyme that catalyzes the conversion of ATP to the second messenger cyclic AMP (cAMP).

Increasing intracellular levels of cAMP leads to the activation of protein kinase A (PKA), a cAMP-dependent protein kinase. Once activated, PKA can then affect downstream messengers that regulate cell proliferation and modulate the cell cycle through the phosphorylation and activation of transcription factors such as cAMP-responsive binding-element protein (CREB) and the RAS/ERK pathway proteins<sup>27</sup>.

The initiation of  $G_q$ -coupled GPCR signaling causes a different intracellular cascade to be activated. Once bound to GTP, active  $G\alpha_q$  dissociates from the  $G\beta\gamma$  heterodimer and activates phospholipase C (PLC). PLC then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ), a membrane-associated phospholipid<sup>28</sup>, into inositol 1,4,5-trisphosphate ( $IP_3$ ) and diacylglycerol (DAG). Free  $IP_3$  then interacts with  $IP_3$  receptors on the endoplasmic reticulum (ER) and stimulates the release of  $Ca^{2+}$  into the cytoplasm. Once bound to cytosolic  $Ca^{2+}$ , DAG stimulates the activation of protein kinase C (PKC)<sup>29</sup>. Activated PKC can then phosphorylate and activate many downstream signaling proteins such as Raf, NF- $\kappa$ B and p38<sup>30</sup>. Receptors that often couple to  $G\alpha_q$  will sometimes display coupling with  $G_{12/13}$  G proteins, the third and least understood of the G protein subfamilies. The initial discovery of the  $G_{12/13}$  subfamily in several vertebrate and

invertebrate species<sup>31, 32</sup> prompted structure function analysis to determine their downstream signaling partners, the most well characterized of those being the RhoGEF signaling cascade<sup>33</sup>. Once activated,  $G\alpha_{12/13}$  will activate RhoGEFs, leading to the exchange of GDP for GTP and activating Rho, a member of the Ras superfamily of GTPases. Activated Rho then contributes to the regulation of actin polymerization and stress fiber formation, affecting cell migration, adhesion and contraction<sup>34</sup>.

Finally, the fourth subfamily of G proteins, the  $G_{i/o}$  subfamily, consists of eight members that propagate a signaling cascade which inhibits AC, leading to decreased levels of cAMP. This lowered cellular level of cAMP leads to a reduction in active PKA and negatively regulates PKA signaling. Interestingly,  $G_{i/o}$  coupled signaling can also affect ERK signaling. Active GTP-bound  $G\alpha_{i/o}$  can interact with a GTPase activating protein (GAP) of the Ras-family protein Rap1 called Rap1GAP, leading to a decrease in the amount of GTP-bound Rap1. The decrease in GTP-Rap1 then leads to the inactivation of the Ras-MAPK-ERK signaling pathway<sup>35</sup>. The inhibitory effect of Rap1 on the ERK signaling cascade remains controversial, however, as Rap1 has also been shown to be involved as an activator of the MAPK/ERK cascade and not involved in its activation as well<sup>36-38</sup>. Despite these conflicting findings, it remains clear that the effects of G protein signaling are wide ranging and play essential roles in mediating important chemosensory processes.

## 1.4 BITTER TASTE RECEPTORS (TAS2RS)

Mammals can detect and interpret five main taste qualities: sweet, salty, sour, umami, and bitter, with a possible sixth modality (fat) having been recently identified<sup>39</sup>. Like all sensory systems, those involved in the detection of taste are thought to have evolved as a tool to enhance survival in new environments and to increase fitness. Sour tastes signify the presence of organic acids while salty taste is generated mostly by sodium, both of which are perceived through the activation of ion channels by proton and Na<sup>+</sup> ion gradient formation across taste receptor cell membranes, respectively<sup>40</sup>. Sweet taste receptors detect carbohydrates and sugars and indicate foods with high caloric content, while umami taste sensations are mediated by the detection of L-amino acids such as monosodium glutamate (MSG)<sup>40</sup>. These sweet and umami ligands are detected by the taste 1 receptor (Tas1R) family of glutamate-like GPCRs, sweet taste sensation being induced by the heterodimerization of the Tas1R2/Tas1R3 monomers while umami taste is detected by Tas1R1/Tas1R3 heteromers<sup>41</sup>.

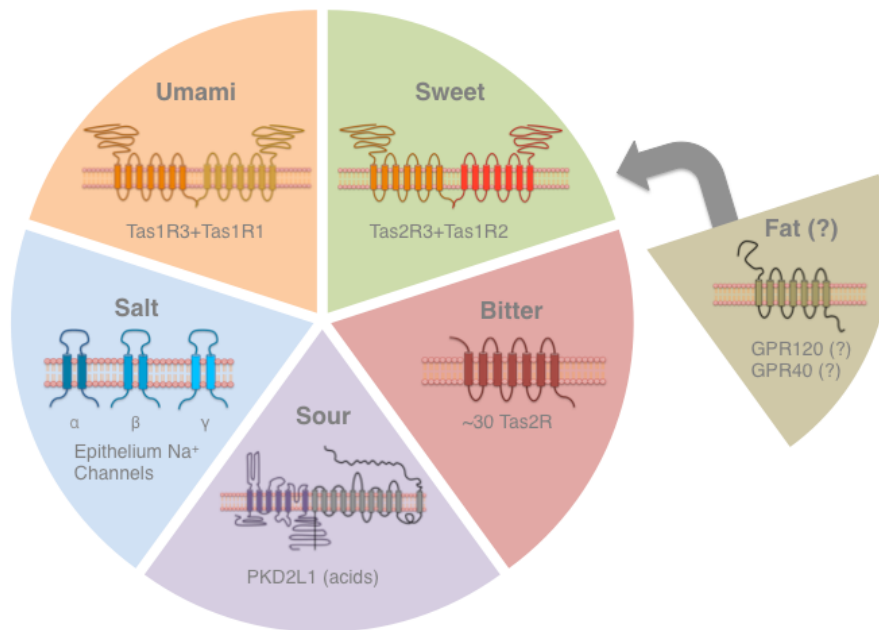
Bitter taste receptors are encoded by the taste 2 receptor gene subfamily (Tas2R) and fall under the frizzled family of GPCRs. As their name implies, they are involved in the perception of natural and artificial bitter substances. The molecular basis of bitter taste detection is thought to have evolved for more practical purposes. Plants often produce poisonous secondary metabolites to protect themselves from ingestion by predators, and mammals, birds and other animals have evolved the ability to detect which plants and plant material are and are not safe to consume. Since a large proportion of poisonous



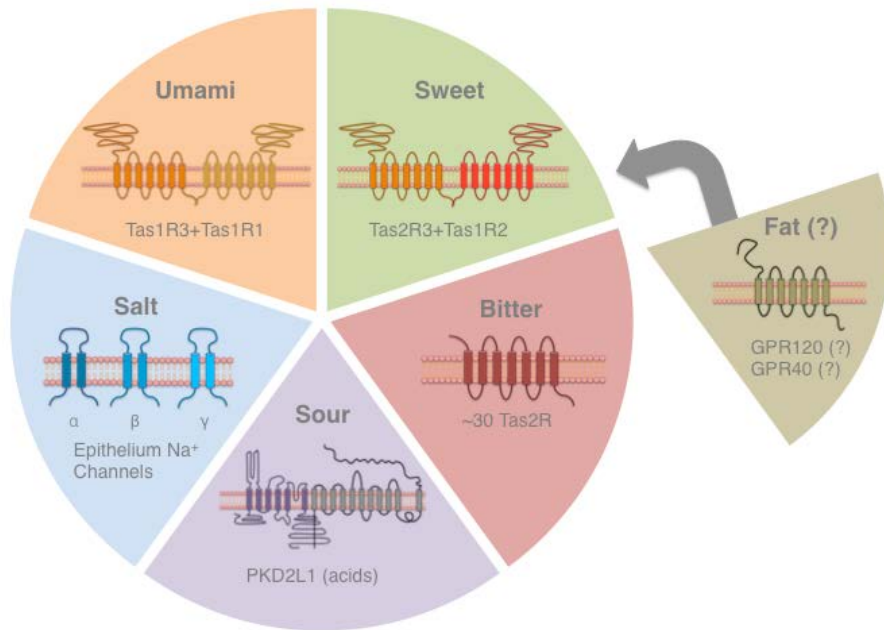
compounds produced by plants are bitter in taste, the ability to sense bitter taste would have proved to be advantageous in avoiding harm. However, the correlation between toxicity and bitterness is complicated. Many bitter compounds, such as those found in coffee, beer and broccoli, are not toxic at concentrations typically consumed, while others even present health benefits such as chemoprotection<sup>42</sup>.

The molecular components involved in the perception of bitter taste had not been known or understood until the early 2000s; until then it had only been hypothesized that there must exist a large family of genes whose products were able to detect bitter compounds, as the chemical entities responsible for evoking bitter taste are structurally diverse<sup>43</sup>. The first biochemical evidence to prove the existence of these molecules came from Chandrashekar *et al.*<sup>44</sup>, who used a heterologous expression system to express three candidate taste receptors, mTas2r5 and mTas2r8 from mice, and hTas2R4 from humans, in modified HEK-293A cells. They showed that cells expressing both the mTas2r5 receptor and the G protein Gα15 responded specifically to cycloheximide, a compound that is exceptionally aversive to mice due to its bitter taste, through a G-protein coupled response resulting in the release of endogenous Ca<sup>2+</sup> from internal stores. Additionally, by assaying a selection of 11 human Tas2Rs, hTas2R4 was found to respond significantly to high levels of denatonium benzoate and 6-n-propyl-2-thiouracil (PROP), and was found to be 70% identical in sequence to the mouse bitter receptor mTas2r8. To determine whether mTas2r5 receptor polymorphisms had any effect on ligand binding or corresponded to the cycloheximide-tasting locus, three previously characterized cycloheximide taster strains (i.e. a mouse strain whose members are sensitive to the taste

of cycloheximide) and one non-taster strain mTas2r5 sequences were isolated and compared to the mTas2r5 sequence in DBA/2J taster and C57BL/6 non-taster mice<sup>44</sup>. It was found that all the taster strains had the same mTas2r5 alleles as the DBA/2J strain and that all the non-tasters harboured the same alleles as those found in the C57BL/6 strain; additionally, the non-taster strains exhibited a change in cycloheximide sensitivity compared to the taster strains, indicating that mTas2r5 is indeed a detector of the bitter ligand cycloheximide. Coupled with the finding that mTas2r5 associates with the taste transduction G-protein gustducin, the authors demonstrated that the TAS2R family of GPCRs is essential in the transduction of bitter taste stimuli.



**Figure 2** **The five taste modalities and their receptors.** Umami, sweet, and bitter taste receptors are members of the GPCR family, while salt taste perception is mediated by epithelial sodium channels and sour taste perception is mediated by acidic compounds acting at the PKD2L1 receptor. The sixth proposed taste modality, fat, is pictured with the GPR120 receptor, which has been implicated in its function along with GPR40. Reprinted with permission from Springer: Encyclopedia of Signaling Molecules (Martin & Dupré, 2016)<sup>45</sup>, Copyright © 2017.



**Figure 2** **The five taste modalities and their receptors.** Umami, sweet, and bitter taste receptors are members of the GPCR family, while salt taste perception is mediated by epithelial sodium channels and sour taste perception is mediated by acidic compounds acting at the PKD2L1 receptor. The sixth proposed taste modality, fat, is pictured with the GPR120 receptor, which has been implicated in its function along with GPR40. Reprinted with permission from Springer: Encyclopedia of Signaling Molecules (Martin & Dupré, 2016)<sup>45</sup>, Copyright © 2017.

## 1.5 EVOLUTION OF TAS2RS IN VERTEBRATE SPECIES

### 1.5.1 Tas2R Repertoires

The dynamic evolution of bitter taste receptors has been documented in the past using comparative genomics and phylogeny-based methods to detect gains and losses across vertebrates, teleost fish, cetaceans, and other species. Feng *et al.*<sup>46</sup> found evidence of massive losses of Tas2R and Tas1R genes in their analysis of six toothed-whale species and five baleen species, such that all three members of the Tas1R gene family and 10 Tas2R receptor genes were pseudogenized, except for Tas2R16 in three baleen whale species. Massive pseudogenization or absence of bitter taste receptor genes have also been found in teleost fish<sup>47</sup>. These discoveries have been explained by a theory positing that vertebrate bitter taste receptor gene evolution was heavily influenced by environmental factors, namely due to the changing feeding behaviors of animals<sup>48</sup>. Thus, herbivorous species of animals would most likely encode and express the largest number of bitter taste receptors as their diets consist of many more bitter molecule-containing foods than omnivores or carnivores. As for the major gene losses in aquatic species such as whales and fish, several other reasons have been presented, among them the notion that the high concentration of sodium in the ocean would conceal any bitter tastant that could present itself to taste receptor cells in the oral cavity and that engulfing food whole may have rendered their taste perceiving machinery obsolete<sup>46</sup>. However, a universal correlation does not exist between dietary habits and *TAS2R* repertoire. For example, the coelacanth species *Latimeria chalumnae* exhibits a large collection of bitter taste

receptors (58)<sup>47</sup>. Coelacanths not only have the largest repertoire of bitter taste receptors among aquatic vertebrates but also one of the largest repertoires among other vertebrates, alongside frogs<sup>49</sup>, guinea pigs<sup>50</sup> and horses with 61, 69 and 55 receptor genes, respectively.

In a recent review, Behrens and Meyerhof<sup>51</sup> outline several reasons as to why these observed correlations between diet and Tas2R repertoire are not always observed. First, some herbivorous species may have developed better methods of digesting bitter or toxic metabolites, potentially leading to losses of bitter taste receptor genes without negatively impacting overall fitness. The next two points relate to the relatively mild correlation between bitterness and toxicity<sup>52</sup>; since not all bitter substances are toxic, it would be expected that mild losses and gains of Tas2R genes might not necessarily influence survival. Additionally, since some bitter compounds can improve the health of animals, seeking behaviors would have had a dynamic effect on shaping their bitter taste receptor gene repertoires. Finally, the ever-expanding list of extra oral bitter taste receptors that have been identified in non-gustatory tissues has muddied scientists' abilities to draw relationships between the two variables, as Tas2Rs may have roles beyond the perception of bitter taste.

### 1.5.2 In- and Between-Species Conservation of Tas2Rs

The human bitter taste receptor family consists of 43 *TAS2R* genes (around 40 % of which are pseudogenes), most which are found in two multigene clusters; 10 gene

sequences on chromosome 7, and 20 on chromosome 12, while only *TAS2R1* is encoded on chromosome 5<sup>53</sup>. Interestingly, the organization of mTas2r sequences in the mouse genome very closely resembles that of humans, where two clusters of mTas2r genes of 10 and 29 sequences are encoded on chromosome 6. The conservation of these motifs has led to the suggestion that the arrangement of *TAS2R* gene clusters was determined prior to the divergence of primates<sup>54</sup>.

The Tas2R family of receptors display a low degree of sequence similarity with Class A/rhodopsin-like GPCRs<sup>55</sup>. As such, they were classified with the Frizzled family of GPCRs; however, in most studies they are reported as distant relatives of classical Class A GPCRs. In contrast to the *TAS1R* family, all *TAS2R* genes contain no spliceosomal introns. Additionally, *TAS2R* gene products exhibit short N-terminal extracellular domains and as such are much shorter in length than their Tas1R counterparts (300 amino acids versus ~800 amino acids). *TAS2R* genes, as with Tas1Rs and salt receptors (epithelial sodium channels or ENaCs), are highly conserved across vertebrates; mouse taste receptor genes in some cases share at least 70% sequence identity with their human counterparts<sup>44</sup>.

**Table 1 Tas2R repertoires in vertebrate species.**

<b>Vertebrate (<i>Genus species</i>)</b>	<b>Number of Tas2R genes</b>			
	<b>Intact</b>	<b>Partial</b>	<b>Pseudogenes</b>	<b>Total</b>
Guinea pig ( <i>Cavia porcellus</i> ) <sup>49</sup>	30	0	39	69
Frog ( <i>Xenopus tropicalis</i> ) <sup>48, 56-58</sup>	51	0	10	61
Coelacanth ( <i>Latimeria chalumnae</i> ) <sup>47</sup>	58	0	1	59
Horse ( <i>Equus caballus</i> ) <sup>48</sup>	21	0	34	55
Lizard ( <i>Anolis carolinensis</i> ) <sup>48</sup>	36	0	14	50
Shrew ( <i>Sorex araneus</i> ) <sup>49</sup>	21	11	13	45
Mouse ( <i>Mus musculus</i> ) <sup>48, 57-61</sup>	35	0	7	42
Rat ( <i>Rattus norvegicus</i> ) <sup>48, 57, 60</sup>	36	0	6	42
Rabbit ( <i>Ochotona cuniculus</i> ) <sup>49</sup>	28	0	13	41
Chimpanzee ( <i>Pan troglodytes</i> ) <sup>49</sup>	26	0	11	37
Bushbaby ( <i>Otolemur garnettii</i> ) <sup>49</sup>	23	4	10	37
Human ( <i>Homo sapiens</i> ) <sup>48, 57-60, 62, 63</sup>	25	0	11	36
Orangutan ( <i>Pongo pygmaeus</i> ) <sup>49</sup>	23	1	12	36
Tree shrew ( <i>Tupaia belangeri</i> ) <sup>49</sup>	16	3	17	36
Rhesus macaque ( <i>Macaca mulatta</i> ) <sup>48</sup>	25	0	10	35
Opossum ( <i>Monodelphis domestica</i> ) <sup>48, 57, 58</sup>	27	1	7	35
Marmoset ( <i>Callithrix jacchus</i> ) <sup>49</sup>	22	1	11	34
Cow ( <i>Bos taurus</i> ) <sup>48, 57, 58</sup>	21	0	13	34
Microbat ( <i>Myotis lucifugus</i> ) <sup>49</sup>	29	1	4	34



Squirrel ( <i>Spermophilus tridecemlineatus</i> ) <sup>49</sup>	19	2	12	33
Gorilla ( <i>Gorilla gorilla</i> ) <sup>49</sup>	24	0	9	33
Naked mole rat ( <i>Heterocephalus glaber</i> ) <sup>49</sup>	18	0	14	32
Manatee ( <i>Trichechus manatus</i> ) <sup>49</sup>	7	0	25	32
Wallaby ( <i>Macropus eugenii</i> ) <sup>49</sup>	16	4	12	32
Pika ( <i>Ochotona princeps</i> ) <sup>49</sup>	14	8	9	31
Tasmanian devil ( <i>Sarcophilus harrisi</i> ) <sup>49</sup>	19	0	11	30
Hedgehog ( <i>Erinaceus europaeus</i> ) <sup>49</sup>	12	5	12	29
Gibbon ( <i>Nomascus leucogenys</i> ) <sup>49</sup>	18	1	8	27
Tarsier ( <i>Tarsius syrichta</i> ) <sup>49</sup>	12	4	11	27
Hyrax ( <i>Procavia capensis</i> ) <sup>49</sup>	14	4	9	27
Panda ( <i>Ailuropoda melanoleuca</i> ) <sup>49</sup>	15	0	11	26
Tenrec ( <i>Echinops telfairi</i> ) <sup>49</sup>	11	4	9	24
Kangaroo rat ( <i>Dipodomys ordii</i> ) <sup>49</sup>	10	7	6	23
Pig ( <i>Sus scrofa</i> ) <sup>49</sup>	15	1	7	23
Megabat ( <i>Pteropus vampyrus</i> ) <sup>49</sup>	15	0	8	23
Mouse lemur ( <i>Microcebus murinus</i> ) <sup>49</sup>	9	4	9	22
Cat ( <i>Felis catus</i> ) <sup>49</sup>	6	11	5	22
Ferret ( <i>Mustela putorius</i> ) <sup>49</sup>	14	0	7	21
Dog ( <i>Canis lupus</i> ) <sup>48, 57, 58</sup>	15	0	6	21
Alpaca ( <i>Vicugna pacos</i> ) <sup>49</sup>	6	6	5	17

Sloth ( <i>Choloepus hoffmanni</i> ) <sup>49</sup>	4	2	9	15
Turtle ( <i>Pelodiscus sinensis</i> ) <sup>49</sup>	11	0	3	14
Armadillo ( <i>Dasyopus novemcinctus</i> ) <sup>49</sup>	5	3	5	13
Dolphin ( <i>Tursiops truncates</i> ) <sup>64</sup>	0	0	10	10
Platypus ( <i>Ornithorhynchus anatinus</i> ) <sup>48</sup>	7	0	3	10
Medium ground finch ( <i>Geospiza fortis</i> ) <sup>49</sup>	6	2	1	9
Zebra finch ( <i>Taeniopygia guttata</i> ) <sup>49</sup>	7	0	1	8
Pufferfish ( <i>Tetraodon nigroviridis</i> ) <sup>48, 57</sup>	6	0	0	6
Cod ( <i>Gadus morhua</i> ) <sup>49</sup>	5	0	0	5
Fugu ( <i>Takifugu rubripes</i> ) <sup>48, 57, 58</sup>	4	0	0	4
Zebrafish ( <i>Danio rerio</i> ) <sup>56-58</sup>	4	0	0	4
Turkey ( <i>Meleagris gallopavo</i> ) <sup>49</sup>	2	0	1	3
Chicken ( <i>Gallus gallus</i> ) <sup>48, 57, 58, 65</sup>	3	0	0	3
Stickleback ( <i>Gasterosteus aculeatus</i> ) <sup>48</sup>	3	0	0	3

## 1.6 THE HUMAN TAS2R REPERTOIRE

The identification of thousands of natural and synthetic bitter substances in the human oral cavity is achieved by around 25 functional members of the human Tas2R gene family. This obvious discrepancy in the ratio of receptors to bitter compounds has raised the question as to how so many different bitter taste stimuli could be perceived by so few

signaling molecules<sup>51</sup>. Two main mechanisms have been put forward to explain this phenomenon; first, as both umami and sweet taste perception is recognized through the heteromerization of Tas1R1/Tas1R3 and Tas1R2/Tas1R3 monomers<sup>66-69</sup>, respectively, it was proposed that perhaps Tas2Rs form heterologous oligomers to account for this discrepancy in ligand-receptor numbers. Kuhn *et al.*<sup>70</sup> set out to determine whether all 325 homo- and heterodimeric combinations of Tas2Rs could exist by using a heterologous expression system in HEK cells and performing a bioluminescence resonance energy transfer (BRET) screen. The binary combination screen demonstrated that most human Tas2Rs form homo and heterodimers *in vitro*, although they did not observe any obvious functionality in those receptors that formed heteromers<sup>70</sup>.

The second mechanism that was proposed to explain the large number of bitter tastant molecules that are perceived by mammalian Tas2Rs was the presence of so called “broadly tuned” Tas2Rs<sup>71</sup>. The first of these identified in humans, hTas2R14, was shown to interact with several structurally diverse bitter tastant molecules including (-)- $\alpha$ -thujone, the active plant metabolite in absinthe, and picrotoxinin, a toxic metabolite from Indian berries<sup>71</sup>. These observations are in contrast with Tas2Rs 16<sup>72</sup> and 38<sup>73</sup>, which exhibit a high specificity for  $\beta$ -glucopyranosides and phenylthiocarbamide (PTC)/PROP-like compounds, respectively. Bitter taste receptors in humans can thus be categorized in four categories: narrowly tuned “specialists” (those whose range of ligand specificity extends to few bitter tastants); broadly tuned “generalists” (such as Tas2R14 and Tas2R10); group-specific receptors (Tas2Rs that recognize only specific classes of molecules such as Tas2R16); and intermediate receptors (Table 2). Therefore, by

retaining a Tas2R repertoire with a wide molecular range, humans can sense an enormous number of structurally diverse bitter compounds<sup>74</sup>.

The diverse tuning abilities of Tas2Rs in humans helped to explain why such a limited number of sensing molecules could interact with so many bitter ligands, although it did not explain the observed in-species variation in response to various bitter taste stimuli. The diverse responses to bitter taste stimuli between populations was thus examined by determining whether there existed allelic variation in the loci of *TAS2R* genes that could account for these differences in taste perception. Kim *et al.*<sup>75, 76</sup> examined 22 out of the 25 identified human *TAS2R* genes in 55 individuals from five geographical areas and performed polymerase chain reaction (PCR) experiments to amplify the open reading frames (ORFs) of these genes. By analyzing these sequences, they identified single nucleotide polymorphisms (SNPs) in the *TAS2R* gene sequences and correlated these to the functional abilities of these Tas2Rs. They demonstrated that each *TAS2R* gene has an average of six SNPs, with some receptors such as TAS2R48 having up to 12 SNP sites, indicating that there exists a wide array of human *TAS2R* haplotypes. Many studies have since identified the repercussions of these polymorphisms such as irreparable changes to the receptor polypeptide chain<sup>73, 77, 78</sup> or minor changes leading to a decreased response to stimulation with bitter ligands<sup>79</sup>, effectively altering Tas2R signaling capabilities.

**Table 2**                      **Categorization of Tas2Rs based on bitter perception ability.**

<b>Group (% of known ligands that interact)</b>	<b>Receptor</b>	<b>Number of known ligands<sup>80</sup></b>	<b>Examples of ligands<sup>80</sup></b>
Broad (~ 30%)	Tas2R10	32	Benzoin, quinine, caffeine, cucurbitacin B, chloramphenicol, erythromycin
	Tas2R14	125	Noscapine, picrotoxinin, (-)- $\alpha$ -thujone, quercetin, genistein, flavone, resveratrol
	Tas2R46	28	Chlorpheniramine, diphenidol, yohimbine, strychnine, hydrocortisone, cnicin
Intermediate (~ 30%)	Tas2R1	35	Humulone, xanthohumol, isoxanthohumol, L-phenylalanine, sodium cyclamate
	Tas2R4	21	Colchicine, denatonium benzoate, azathioprine, quassin
	Tas2R7	9	Papaverine, chloroquine, cromolyn, H. g.-12 ( <i>Hoodia gordonii</i> )
	Tas2R8	3	Parthenolide, chloramphenicol, denatonium benzoate
	Tas2R30	10	Absinthin, cascarillin, diphenidol, andrographolide
	Tas2R31	8	Famotidine, acesulfame K, aloin, saccharin
	Tas2R39	78	Thiamine, acetaminophen, coumestrol, flavone, resveratrol
	Tas2R40	11	Humulone, colupulone, dapsone, adhumulone, diphenidol
	Tas2R43	16	Arglabin, caffeine, helicin, amarogentin, aristolochic acid
Narrow (~ 10%)	Tas2R3	1	Chloroquinine
	Tas2R5	1	1,10-phenanthroline
	Tas2R13	2	Diphenidol, denatonium benzoate
	Tas2R20	2	Diphenidol, cromolyn
	Tas2R41	1	Chloramphenicol
	Tas2R50	2	Amarogentin, andrographolide
Group-specific	Tas2R16	10	Arbutin, diphenidol, sodium benzoate, D-salicin, phenyl $\beta$ -D-glucopyranoside

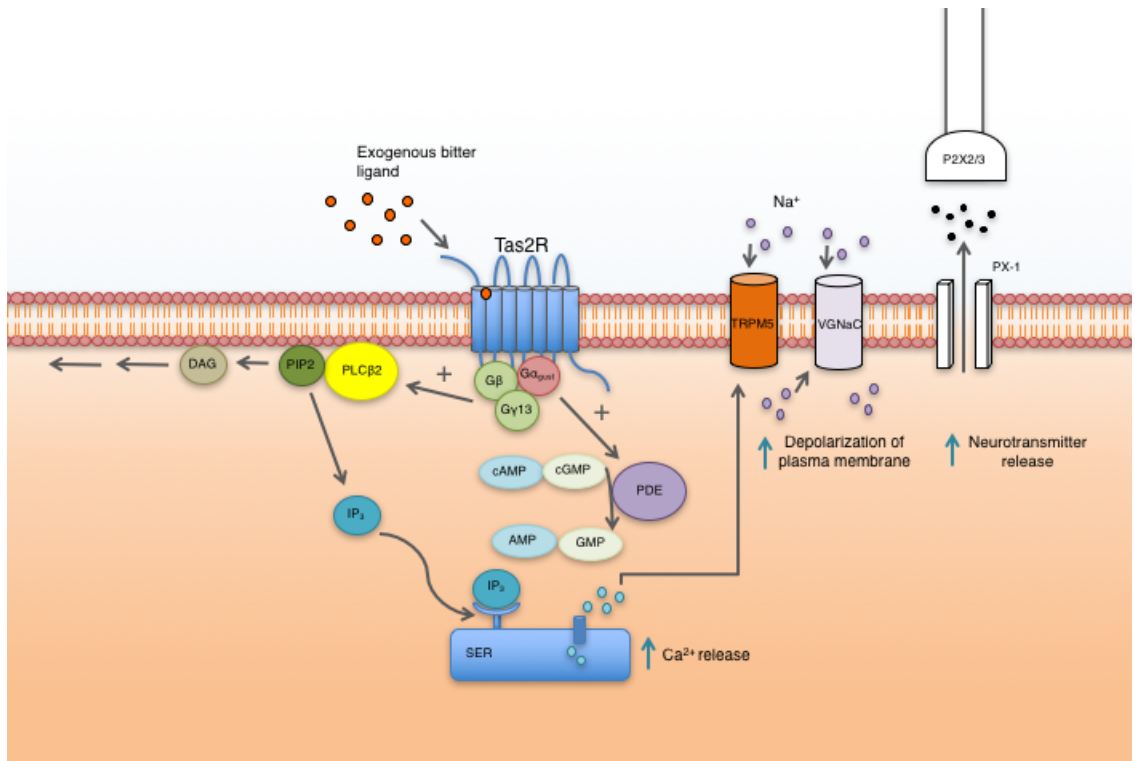
(~ 20%)	Tas2R38	21	Limonin, phenylthiocarbamide (PTC), propylthiouracil (PROP), N-methylthiourea
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## 1.7 TAS2R SIGNALING

The first protein identified during initial research into the molecular basis of taste in the oral cavity was the  $G\alpha$ -subunit gustducin ( $G\alpha_{\text{gust}}$ )<sup>81</sup>. McLaughlin *et al.*<sup>81</sup> first identified gustducin through amplifying and cloning  $G\alpha$  cDNAs from a library of taste tissue cDNAs, and found that not only was  $\alpha$ -gustducin universally expressed in circumvallate, foliate and fungiform taste papillae, but that it most closely resembled the  $\alpha$ -transducin rod and cone photoreceptor G proteins, implicating their role in taste transduction. Tas2Rs are thus almost without exception expressed in  $G\alpha_{\text{gust}}$  containing cells.

The involvement of  $G\alpha_{\text{gust}}$  in the transduction of bitter taste signals is crucial for full activation and signaling to occur, as demonstrated through the use of mouse knockout models demonstrating reduced response to bitter compounds in the absence of  $G\alpha_{\text{gust}}$ <sup>82</sup>. However, lacking the  $G\alpha_{\text{gust}}$  subunit does not limit the potential for GPCR activation, as knockout does not completely abolish bitter taste sensation. Therefore, bitter taste potentiation may still occur with the help of other  $G\alpha$  protein subunits expressed in TRCs. This finding has raised the question of whether  $G\alpha_{\text{gust}}$  is simply favored due to relative abundance in taste tissues, whether different Tas2Rs are selective for G-protein subunits to become fully activated, or whether other biological factors such as spatial arrangement in taste cells exist that may play a role in their coupling to bitter taste receptors<sup>83</sup>.

Co-localization and mouse knockout studies were performed early in the elucidation of bitter taste receptor signaling to determine the factors necessary for proper signal transduction of bitter taste stimuli. For signaling to occur, the formation of a heterotrimeric G-protein complex between  $\alpha$ -gustducin and G $\beta$ 3 and G $\gamma$ 13 occurs the most often, while some trimers are comprised of G $\beta$ 1<sup>83</sup>. Tas2R stimulation and activation of the G protein heterotrimer leads to the dissociation of G $\alpha_{\text{gust}}$  and G $\beta$ 3 $\gamma$ 13, the latter of which activates PLC $\beta$ 2. Active PLC $\beta$ 2 then causes an increase in cellular levels of IP<sub>3</sub> and DAG via the breakdown of PIP<sub>2</sub>. Free IP<sub>3</sub> then binds to IP<sub>3</sub> receptors on the ER and stimulates the release of Ca<sup>2+</sup> into the cytosol. As the cytoplasmic concentration of Ca<sup>2+</sup> increases, free Ca<sup>2+</sup> binds a member of the transient receptor potential cation channel protein M family (TRPM5), whose induction generates a depolarization across the taste receptor cell membrane through the influx of Na<sup>+</sup> ions. This influx of Na<sup>+</sup> leads to activation of voltage gated sodium channels (VGNaCs) on the cell membrane, allowing further depolarization of the membrane and an eventual action potential<sup>84</sup>. ATP, now recognized as the primary taste cell neurotransmitter, is then released from the taste receptor cell and acts on ionotropic purigenic receptors P2X2 and P2X3 on the afferent terminals of gustatory nerves enervating to the brain<sup>85</sup>. Additionally, the G $\alpha_{\text{gust}}$  subunit activates phosphodiesterase (PDE) resulting in a decrease in cellular cyclic nucleotide monophosphates (cNMPs), such as cAMP and cGMP<sup>86</sup>; however, the exact reason for which these changes in cNMPs occur in taste receptor cells and the role of cNMP signaling in G $\alpha_{\text{gust}}$ -coupled taste transmission has not yet been determined<sup>83</sup>.



**Figure 3** **Tas2R signaling in the oral cavity.** Stimulation of Tas2Rs in the oral cavity with exogenous bitter ligands leads to the activation of the heterotrimeric G-protein complex and the dissociation of  $G\alpha_{gust}$  from  $G\beta\gamma_{13}$ .  $G\beta\gamma_{13}$  activates PLC $\beta$ 2 at the plasma membrane, which catalyzes the breakdown of PIP $_2$  to DAG and IP $_3$ . IP $_3$  then binds to IP $_3$  receptors on the smooth ER, leading to release of intracellular  $Ca^{2+}$ . Increases in  $Ca^{2+}$  activate the TRPM5 channel and cause influx of  $Na^+$ , which leads to further depolarization of the membrane through voltage gated sodium channels (VGNaCs). Upon depolarization, ATP, the primary neurotransmitter, is released through PX-1 and stimulates P2X2/3 receptors on afferent terminals of nearby gustatory nerves. Figure modified and reprinted with permission from Springer: Encyclopedia of Signaling Molecules (Martin & Dupré, 2016)<sup>45</sup>, Copyright © 2017.



## 1.8 TAS2R EXPRESSION

### 1.8.1 Expression in the Oral Cavity

Tas2R expression in the oral cavity is restricted to the surface of gustducin-expressing type II taste receptor cells (TRCs) lining tongue microvilli<sup>87</sup>, and are thus not expressed in sweet or umami receptor expressing cells<sup>88</sup>. Neurophysiological studies have lent credence to two possible modes of expression of Tas2Rs in the mouth. TRCs may co-express multiple Tas2Rs, such that most or all receptor subtypes are expressed in any given Tas2R-positive cell, or they may exhibit minimal Tas2R repertoires and thus express only a few receptor subtypes. *In situ* hybridization and genetically engineered mouse experiments performed by Adler *et al.*<sup>43</sup> and Mueller *et al.*<sup>89</sup> have provided support for the “broadly tuned” model of TRCs while others have found that Tas2Rs may be heterogeneously expressed in bitter TRCs<sup>90,91</sup>. The latter of these two postulations has become the more accepted of the two, as mRNA analysis of human and mouse tongue tissue has supported a heterogeneous distribution of Tas2Rs<sup>92,93</sup>.

### 1.8.2 Expression in the Gastrointestinal Tract

Interestingly, the expression of bitter taste receptors is not restricted solely to the oral cavity. The notion that other tissues along the digestive tract may contain cells expressing functional chemosensory proteins was originally proposed by Fujita<sup>94</sup>, who observed that the enteroendocrine cells of the gut epithelium, whose microvilli-lined apical processes

interact with incoming foodstuffs, structurally and functionally resembled taste receptor cells in the mouth. Thus, in 1996, Höfer *et al.*<sup>95</sup> attempted to demonstrate that enteroendocrine cells express molecules important in taste recognition, and were the first to identify extra-orally expressed  $\alpha$ -gustducin in TRC-like brush cells of the stomach and intestine. Further co-localization studies and characterization of mouse stomach cells expressing  $\alpha$ -gustducin revealed that  $\alpha$ -gustducin is expressed on solitary chemosensory cells (SCCs) as well as glucagon-like peptide 1 (GLP-1) producing cells in the small intestine<sup>87, 96</sup>. As SCCs expressing  $\alpha$ -gustducin were found to be spatially located near ghrelin/serotonin-releasing and GLP-1-releasing cells, it was suggested that SCCs may act as chemosensory mediators of appetite regulation by communicating with these adjacent secretory cells. Interestingly, treatment of NCI-H716 human enteroendocrine cells, or intragastric administration of the hTas2R4/hTas2R10-agonist denatonium benzoate and extracts from the root of the Korean gentian plant (*Gentiana scabra*) in mice, lead to  $\alpha$ -gustducin-dependent increases in cholecystokinin (CKK) and GLP-1 release<sup>77, 97-100</sup>. Additionally, intragastric administration of a mixture of bitter compounds in mice and rats lead to modest increases in ghrelin, causing short-lived increases in food intake followed by prolonged decrease in feeding behaviour<sup>101</sup>.

These observed changes in orexigenic and anorexigenic peptide release lead to the characterization of Tas2Rs in not only cells of the intestinal epithelium, but also those of the smooth muscle lining the gut, as the observed changes in feeding behaviours in mice were associated with delayed gastric emptying. Administration of denatonium benzoate to muscle strips isolated from the fundi of mice induced canonical Tas2R pathway-

dependent smooth-muscle contraction and inhibited gastric emptying in mice<sup>102</sup>.

Additionally, healthy patient volunteers who underwent intragastric nutrient infusion post treatment with denatonium benzoate experienced increased intragastric pressure as well as increased satiation compared to those pretreated with placebo<sup>102</sup>. The implications of these findings are intriguing as they suggested another role for Tas2Rs other than the neurological transmission of bitter taste stimuli in the oral cavity despite still being linked to the direct consumption of bitter-tasting foods. These experiments indicated that Tas2Rs expressed on gut epithelium and gut smooth muscle cells may be acting to hormonally and muscularly regulate dietary habits. Coupled with mounting evidence suggesting that bitter taste receptor expression can be negatively and positively regulated by dietary interventions<sup>103-105</sup>, Tas2Rs could prove to be viable pharmaceutical targets for the treatment of GI disorders.

### 1.8.3 Expression in the Respiratory Tract

Upon the discovery that Tas2Rs are expressed on SCCs and ciliated cells of the gut, several groups sought to characterize their expression on SCCs of the airway epithelium. As a result, bitter signaling mediators ( $\alpha$ -gustducin<sup>106-108</sup>, TRPM5<sup>109</sup>, IP<sub>3</sub> receptors<sup>108</sup> and PLC $\beta$ 2<sup>108, 110</sup>) and several Tas2Rs have since been characterized in the SCCs of multiple species and their physiological role in the airway has been of intrigue to many. Activated hTas2Rs/mTas2rs appear to display interesting roles in both innate airway immunity and cell autonomous responses. PLC-dependent calcium release and trigeminal nerve stimulation was observed when denatonium benzoate was applied to isolated mouse

SCCs from nasal epithelium, as was a cessation of breathing upon application to anaesthetized rats<sup>107, 111</sup>. A different response was observed in human nasal SCCs responsive to denatonium benzoate expressing the bitter taste receptor Tas2R47, where bitter agonist stimulation lead to a “calcium wave” which proceeded through gap junctions to other epithelial cells in the nose and stimulated release of antimicrobial peptides involved in preventing increased bacterial colonization<sup>112</sup>. Additionally, nasal SCC TasRs appeared to be responsive to acyl-homoserine lactones (AHLs) released by Gram-negative bacteria and their activation prevented further bacterial colonization by promoting pro-inflammatory neuropeptide release from mast cells<sup>113, 114</sup>.

In addition to their role in innate immunity of the upper airway, several studies have elucidated the involvement of Tas2Rs in airway smooth muscle contraction. An initial screen<sup>115</sup> elucidating the expression of Tas2Rs on human airway smooth muscle cells prompted Deshpande *et al.*<sup>116</sup> to characterize their potential as new targets for the treatment of asthma. Bitter taste receptors expressed on airway smooth muscle were not only functional and signalled in a calcium-dependent fashion, but were paradoxically able to induce a level of bronchial relaxation that was three times greater than a commercially available  $\beta_2$ -agonist<sup>116</sup>. The efficacy of Tas2Rs in comparison to  $\beta_2$ -adrenergic induced bronchodilation has been called into question by some and several groups have reported conflicting results describing the degree of Tas2R agonism on smooth muscle relaxation. Despite these conflicting accounts of their ability to out-perform conventional  $\beta_2$ -agonists, Tas2R-agonists have been recognized by the majority as having a bona fide

therapeutic potential, perhaps most effectively as a combination therapy with existing  $\beta_2$  therapies.

#### 1.8.4 Expression in Other Extra-Oral Tissues and Disease States

Tas2Rs have been discovered in many other non-gustatory tract tissues such as the heart<sup>103</sup>, the brain<sup>117</sup> and the testis<sup>118</sup>, further suggesting that their neurostimulatory role in the oral cavity does not represent the entirety of their signaling abilities. Additionally, their differential expression in pathologies such as hypo- and hyperthyroidism<sup>119</sup>, Schizophrenia<sup>120</sup> and Parkinson's disease<sup>121</sup> suggests that Tas2Rs could prove to be valuable drug targets in multiple diseased states<sup>122</sup>.

The expression of bitter taste receptors has been documented in various cancers as well. Carrai *et al.*<sup>123</sup> forged the first link between Tas2Rs and cancer by investigating whether genetic variability in *TAS2R38* correlated with the risk of developing colorectal cancer in 1203 German and Czech colorectal cancer cases. Two SNPs of the *TAS2R38* gene, the functional PAV and non-functional AVI haplotypes, are the most common in European and North American populations and are associated with the ability to perceive 6-propyl-2-thiouracil, or PROP, as bitter<sup>124</sup>. As the PAV "taster" *TAS2R38* haplotype mediates strong responses to bitter taste stimuli, and low bitter vegetable intake can increase colorectal cancer risk, it was proposed that "tasters" may exhibit a higher risk of developing colorectal cancer as a possible consequence of avoiding bitter antioxidant-rich vegetables. No association between CRC risk and SNP haplotype (PAV or AVI) was

observed; however, when attempting to observe any correlation between *TAS2R38* diplotypes and cancer risk (PAV/PAV, PAV/AVI and AVI/AVI), Carrai *et al.* found that the “non-taster” AVI/AVI genotype was associated with increased risk of developing colorectal cancer<sup>123</sup>. These results demonstrated that aversion to bitter tasting foods may not pose as high of a risk on the development of colorectal cancer as those posed by the expression of a non-functional bitter taste receptor, suggesting the importance of *Tas2R38* signaling in the colon.

More recently, *Tas2Rs* have been identified in breast cancer and pancreatic cancer cells. *Tas2R4* expression was downregulated by 20–30 % in the breast cancer cell lines MDA-MB-231 and MCF-7 when compared to the noncancerous cell line MCF-10A. Functional calcium assays were conducted using quinine, dextromethorphan, and phenylthiocarbamide, showing that although reduced in number, *Tas2Rs* are functional in breast cancer cells<sup>125</sup>. The expression of another taste receptor, *Tas2R38*, was identified in lipid droplets in pancreatic ductal adenocarcinoma tumour biopsies as well as in a pancreatic cancer-derived cell line<sup>126</sup>. Stimulation of pancreatic *Tas2R38* by phenylthiourea or N-acetyldodecanoyl homoserine was found to induce activation of p38 and ERK-1/2 while upregulating the expression of nuclear factor of activated T-cells c1 (NFATc1). As active NFATc1 binds to the interleukin-2 promoter in the nucleus during T-cell activation<sup>127</sup> and has been found to play roles in metastatic processes such as tumour cell migration<sup>128</sup> and tumour angiogenesis<sup>129</sup>, exploring its relationship with activated bitter taste receptors in various cancers would be an interesting avenue to explore the relationships between bitterness, immunity and cancer therapies. The

discovery of functionally active Tas2Rs in these cancers is therefore intriguing. Expanding the list of pathologies in which functional Tas2Rs are expressed is thus warranted, as their links to pro- and anti-apoptotic pathways in these tissues could help in developing novel small molecule anti-cancer therapies aimed at modulating cancer immune responses or apoptosis.

## **1.9 OVARIAN CANCER**

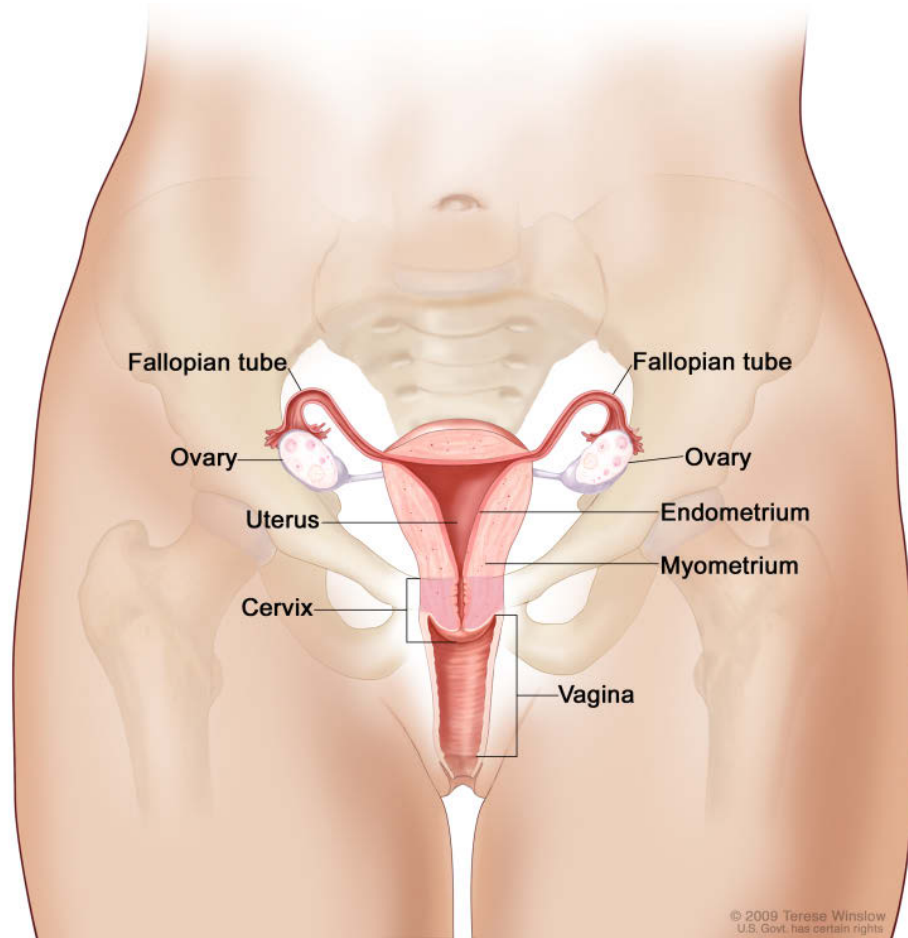
### **1.9.1 Overview and Pathological Classification**

Ovarian cancer is the deadliest gynecological cancer and the seventh most common cancer among women<sup>130</sup>. It is estimated that nearly 240,000 new cases of ovarian cancer are recorded each year worldwide, and that the estimated death-toll is 152,000 annually<sup>131</sup>. The low 5-year relative survival rate of ovarian cancer (~29%) is typically a result of its primary presentation as metastatic disease. This observation, coupled with the fact that there are currently no reliable screening methods available to detect early stage carcinomas<sup>132</sup>, has impacted the development of new surgical or therapeutic strategies and has led to no significant reduction in ovarian cancer-related mortality over the last two decades<sup>133</sup>.

Ovarian cancer can be classified as being of epithelial, stromal or germline cell origin. As nearly 90% of all documented cases of ovarian cancer tend to be of epithelial cell origin<sup>134</sup>, most case studies and new therapies tend to be directed at these cancers.

Epithelial ovarian cancers (EOCs) are then subdivided into several histotypes which depend on their morphological characteristics<sup>135</sup>, sites of origin<sup>136</sup> and genetic and expression profiles<sup>137</sup>. Malignant EOCs have thus been classified as being either high-grade serous (HGSEOC, accounting for 70% of EOCs), low-grade serous (LGSEOC, <5%), endometrioid (10%), clear cell (10%) and mucinous (3%)<sup>130, 135, 136</sup>. It has become increasingly accepted that most invasive ovarian tumours do not arise from precursor lesions in the ovarian epithelium, but rather originate in other tissues and migrate to the ovary secondarily. The “serous” designation thus classifies tumours thought to be of fallopian tube epithelial origin<sup>138-145</sup> often modeled in laboratory conditions with the cell lines OVCAR4 and OVCAR8, while those of endometrioid and clear-cell character are thought to arise from endometrial cysts retroactively transplanted into the ovaries<sup>133, 146-149</sup>, represented by the cell lines IGROV1 and SKOV3, respectively.





**Figure 4**     **The female reproductive system.** Anatomical representation of the major areas of the female reproductive tract. High grade serous epithelial ovarian cancers (HGSEOCs) originate in the epithelium of the Fallopian tube and then establish themselves in the ovary, while endometrioid and clear cell ovarian cancers originate in the endometrium/the uterine epithelium and are established in the ovary via retrograde menstruation. Figure modified and reprinted with permission from Terese Winslow Medical and Scientific Illustration, Copyright © 2017.

### 1.9.2 Natural Bitter Compounds and Ovarian Cancer

The standard of treatment for EOCs usually consists of surgical resection alongside platinum-based and taxane chemotherapies such as cisplatin, a purine base DNA crosslinking agent that interferes with DNA repair and induces DNA damage<sup>150</sup>, and paclitaxel, a microtubule-stabilizing compound that induces mitotic arrest in actively dividing cells<sup>151</sup>, respectively<sup>152</sup>. Quite often, chemoresistance and relapse occur in EOC after the administration of these first line therapies<sup>153</sup>; thus, identifying new chemotherapeutic targets and strategies are essential to improve patient outcomes. Because of the frequent and undesirable toxic side effects of most small molecule antineoplastic compounds in clinical trials, recent efforts have been aimed at the identification of nutraceutical agents or traditional medicines. Natural bitter compounds have been shown in several cancers, including EOCs<sup>154</sup>, to induce cancer cell death; extracts from the bitter melon *Momordica charantia* were shown to inhibit cell progression and proliferation and reduce multiple drug resistance (MDR)-associated resistance in colon cancer<sup>155, 156</sup>, to arrest prostate cancer cells in S phase, and to delay cell cycle progression in a mouse model of human prostatic adenocarcinoma<sup>157</sup>, to induce apoptosis in breast cancer cells<sup>158</sup>, and both inhibit tumour formation and improve the efficacy of cisplatin-based chemotherapy in EOC<sup>154</sup>. Noscapine, a phthalide isoquinoline non-narcotic alkaloid derived from the opium poppy *Papaver somniferum*, sensitized a cisplatin-resistant derivative of the SKOV3 clear cell EOC cell line to platinum-based chemotherapy by increasing the number of cells in the G<sub>2</sub>/M phase of the cell cycle and induce caspase-mediated apoptosis<sup>159</sup>. The underlying molecular mechanisms of many

nutraceutical agents with anti-cancer activity remain poorly defined, and some of their activity could be dependent on Tas2Rs expressed in these tissues. Noscaphine is an agonist of the human bitter taste receptor Tas2R14, and along with other bitter ligands, demonstrates effects on cancer cells<sup>159-162</sup>; but whether its effect on EOCs is mediated through extra-oral expression of Tas2R14 is thus far unknown.

## 1.10 OBJECTIVES

As several natural bitter products have been shown to influence the growth and death of ovarian cancer cells, the objectives of this study were to:

1. Characterize the expression of several Tas2Rs at the mRNA and protein level in EOC cells and primary tissue samples.
2. Determine the functional consequences of Tas2R activation by a bitter phytochemical in EOC cells.

It was hypothesized that ovarian cancer cells are affected through Tas2R-mediated signaling.

## CHAPTER 2 METHODS

### 2.1 CELL CULTURE

Ovarian cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) except OVCAR4, obtained from NCI-Frederick Cancer DCTD Tumor/Cell Line repository, Frederick, MD, USA. Cells were grown in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> and 37°C). OVCAR8, IGROV1, SKOV3, and HEC-1a cells were cultured in DMEM (Sigma-Aldrich; Oakville, ON) supplemented with 10% fetal bovine serum (Thermo Fisher; Waltham, MA) and 10% penicillin-streptomycin (Thermo Fisher). OVCAR4 cells were cultured in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 10% penicillin-streptomycin. Institutional approval for research with human materials was received prior to the initiation of these studies (University of Manitoba, #HS12920), and primary human EOC cell samples were obtained after receiving Informed Consent. Primary human EOC cells were isolated from ascites fluid obtained from patients with stage III or IV high grade serous adenocarcinoma, and were isolated and grown as previously described<sup>163, 164</sup>. The FT240 normal fallopian tube cell line was obtained from the Drapkin laboratory (Penn Ovarian Cancer Research Center; Philadelphia, PA) and grown as previously described<sup>165</sup>.

## **2.2 RNA SAMPLES**

Ovarian cell line total RNA was isolated from OVCAR8, IgROV1, SKOV3 and OVCAR4 cell lines. Total RNA from EOC cells and a papillary serous ovarian cystadenocarcinoma from a 53-year-old Caucasian female (Clontech; Mountain View, CA) was extracted using a TRIzol/100% isopropanol protocol (Thermo Fisher). Human fallopian tube total RNA from a single 47-year-old female donor and human uterine total RNA from a 57-year-old female donor total RNA was isolated using a modified guanidinium thiocyanate method (Agilent Technologies; Santa Clara, CA). HEC-1a cell line total RNA was isolated using the RNeasy Plus kit (QIAGEN).

## **2.3 RNA EXTRACTION AND CDNA SYNTHESIS**

Oligo(dT)12-18 primers, Superscript II Reverse Transcriptase (200 U/ $\mu$ L) and 10mM dNTP mix were purchased from Invitrogen (Carlsbad, CA). A first-strand cDNA synthesis step was performed on total RNA isolated from ovarian cell line, ovarian tumour tissue, uterine tissue and fallopian tissue mRNA samples as per manufacturer's recommendations with a single incubation step at 42°C for 50 minutes, followed by an inactivation step at 72°C for 15 minutes.

## 2.4 QUANTITATIVE PCR (QPCR)

A Taqman® Probe-Based Gene Expression Assay was performed using Taqman® probes for Tas2R1 (assay ID: Hs00251930\_s1), Tas2R4 (assay ID: Hs00249946\_s1), Tas2R10 (assay ID: Hs00256794\_s1), Tas2R14 (assay ID: Hs00256800\_s1), Tas2R38 (assay ID: Hs00604294\_s1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, assay ID: Hs03929097\_g1). qPCR was performed on a LightCycler96 according to manufacturer recommendations and analyzed using LightCycler96 software (Roche, Basel, Switzerland). The  $2^{-\Delta\Delta C_T}$  method was used to establish relative gene expression levels compared to GAPDH<sup>166, 167</sup>.

## 2.5 ANTIBODIES

Tas2R1 (OSR00153W), Tas2R4 (OSR00153W), and Tas2R14 (OSR00161W) rabbit polyclonal antibodies used for Western blotting were purchased from Osenses (Keswick, Australia). Caspase-3 p11 mouse monoclonal antibody (sc-271759) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Bcl-XL rabbit monoclonal antibody (2764S) was purchased from Cell Signaling Technology (Danvers, MA).  $\beta$ -actin (ab8226) and  $\beta$ -tubulin (ab6046) mouse monoclonal antibodies was purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG-HRP (sc-2005) polyclonal antibodies were purchased from Santa Cruz Biotechnology.

## **2.6 IMMUNOHISTOCHEMISTRY/IMMUNOCYTOCHEMISTRY**

Paraffin-embedded high grade serous ovarian cancer tissue samples (EOC75, 88, and 127) were obtained from the Manitoba Ovarian Biobank Program at the Manitoba Tumor Bank. Samples were processed in a Leica Bond Rx autostainer using the Leica Bond Polymer Refine to detect expression of Tas2R14 (1:2000 dilution). Images were acquired with a Leica DM4000B microscope with a Leica DFC480 camera.

For immunocytochemistry, cells were grown on glass coverslips. Cells were rinsed with PBS and fixed in fresh 2% paraformaldehyde for 5 min at room temperature, followed by 2 washes with PBS. Cells were permeabilized and blocked with 5% goat serum in PBS containing 0.5% Triton X-100. Slides were incubated with primary Tas2R14 antibody (1:1000 dilution) overnight at 4°C in a humidified chamber. Cells were subsequently washed 3 times and incubated with secondary antibody (Alexa-488, goat anti-rabbit IgG; Invitrogen, A11008) for 1 h at room temperature. Nuclei were stained with Hoechst 33342. Cell were visualized using a Zeiss Axio Imager Z2 microscope with AxioVisio Rel.4.8.2 software.

## **2.7 siRNA TRANSFECTION**

Negative control siRNA (ID 1027310), Tas2R1-specific siRNA (SI03044734, SI03032617, SI02643690, SI02643676) and Tas2R4-specific siRNA (SI03119144, SI02642535, SI00117789, SI00117782) were purchased from Qiagen (Hilden, Germany).



Tas2R14-specific siRNA (s27146) was purchased from Ambion (Burlington, ON). X-tremeGENE HP DNA Transfection Reagent was purchased from Roche (Basel, Switzerland). Semi-confluent plates (~80%) were transfected with siRNAs per manufacturers recommendations in a 1:5 ratio (siRNA:X-tremeGENE HP) for 24 hours prior to cell lysis for Western blot analysis or treatment with Tas2R-specific ligands.

**Table 3** Sequences of siRNAs used. Manufacturer is identified below the siRNA ID in brackets.

siRNA ID	Sense strand	Antisense strand
Negative (-) control siRNA (Qiagen)	5'-UUCUCCGAAC GUGUCACGUTT-3'	5'-ACGUGACACGU UCGGAGAATT-3'
Hs_TAS2R1_5 (Qiagen)	5'-GGACACUCUC UCAUCUUAATT-3'	5'-UUAAGAUGAG AGAGUGUCCAG-3'
Hs_TAS2R1-7 (Qiagen)	5'-ACGUAAAUGU GAUUGUUAUTT-3'	5'-AUAACAAUCA CAUUAACGUAG-3'
Hs_TAS2R1_8 (Qiagen)	5'-GCAAUUGAU GCUAGAGUATT-3'	5'-UACUCUAGCA UCAAUUUGCTT-3'
Hs_TAS2R4_1 (Qiagen)	5'-GGUUUGUGAC CUUGCUCUATT-3'	5'-UUGAGCAAGG UCACAAACCAG-3'
Hs_TAS2R4_2 (Qiagen)	5'-GGACAUUCUGU UCUCAUUATT-3'	5'-UAAUGAGAAC AGAAUGUCCTG-3'
Hs_TAS2R4_6 (Qiagen)	5'-UGUUUAUUAC AGUGGUCAATT-3'	5'-UUGACCACUG UAAUAAACAGA-3'

Hs_TAS2R4_7 (Qiagen)	5'-CUAUGAAGCU GAUGGUCUATT-3'	5'-UAGACCAUCAG CUUCAUAGCA-3'
Silencer® Select vs TAS2R14 (Ambion)	5'-UGAUAAACAU CCAUAUAAATT-3'	5'-UUUAUAUGGAU GUUUAUCAGT-3'
TAS2R38HSS108754 (Invitrogen)	5'-CCAGAUGCUCU GGGUAUUAUUCUU-3'	5'- AAGAAUAAUAC CCAGGAGCAUCUGG- 3'
TAS2R38HSS108755 (Invitrogen)	5'-GGCACAUGAGG ACAAUGAAGGUCUA-3'	5'- UAGACCUUCAU UGUCCUCAUGUGCC- 3'
TAS2R38HSS108756 (Invitrogen)	5'-CCUACUGAUUCU GUGGCGGACAAA-3'	5'-UUUGUCGCGCCAC AGAAUCAGUAGG-3'

## 2.8 CELL LYSIS AND WESTERN BLOTTING

Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1 cOmplete™ EDTA-free protease inhibitor cocktail tablet (Roche). BSA-coated beads (Protein A-Sepharose® lypholised powder from *Staphylococcus aureus*, Sigma-Aldrich) and 10% DNase I (Sigma-Aldrich) were added to remove nucleic acid and organellar material from the sample. Lysates were mixed 50:50 with 2X Laemmli Buffer and 1X 2-mercaptoethanol (final concentration) (Bio-Rad Laboratories, Hercules, CA). Samples were run on a 10%

polyacrylamide SDS-PAGE gel and transferred to nitrocellulose membranes before being blocked in a 10% skim milk powder/PBS solution for 60 minutes, and incubated overnight at 4°C with their respective primary antibodies. Chemiluminescence was performed on nitrocellulose membranes using Western Lightning® Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer, Waltham, MA) before exposing them to x-ray film and development.

## **2.9 TAS2R LIGANDS**

Colchicine [(S)-N-(5,6,7,9- tetrahydro-1,2,3,10-tetramethoxy-9 oxobenzol[a]heptalen-7-yl) acetamide] and noscapine [S(R\*,S\*)-6,7-Dimethoxy-3-(5,6,7,8-tetrahydro-4-methoxy-6-methyl-1,3- dioxolo[4,5-g]-isoquinolin-5-yl)-1(3H)-isobenzofuranone] were purchased through Cedarlane (Burlington, ON) from MP Biomedicals (Ca. No's. 05208170 and 02153558, respectively; Santa Ana, CA). 6-propyl-2-thiouracil [2,3-Dihydro-6-propyl-2-thioxo-4(1H)-pyrimidinone] was purchased from Sigma-Aldrich.

## **2.10 CALCIUM MOBILIZATION ASSAY**

The Fluo-4 NW Calcium Assay kit (Ref. No. F36206) was used to detect changes in intracellular calcium release (Thermo Fisher). Cells were seeded at a density of 25,000 cells/well and left overnight in a CO<sub>2</sub> incubator. Growth medium was removed and Fluo-4 NW dye mixed in assay buffer (1X HBSS, 20 mM HEPES) was added to the cells. After a 30-minute incubation at 5% CO<sub>2</sub>/37°C and 30 minutes at 25°C, calcium

mobilization was assessed immediately after stimulation of Tas2R4, Tas2R14 and Tas2R38 with colchicine (100  $\mu$ M) noscapine (100  $\mu$ M) and PROP (10  $\mu$ M) respectively. Fluorescence was recorded using an EnVision Multilabel Reader (software version 1.12, product no. 2104-0010A; PerkinElmer).

**Table 4** **Bitter ligands and their known molecular targets.** Molecular weights, chemical formulas and effective concentrations\*.

Ligand Name	IUPAC Systemic Name	Molecular Weight	Tas2R Targetted	Effective Concentration ( $\mu$ M)*
Colchicine	(S)-N-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzol[a]heptalen-7-yl) acetamide	399.437	Tas2R4 Tas2R39 Tas2R46	100 3000 300
Noscapine	S(R*,S*)-6,7-Dimethoxy-3-(5,6,7,8-tetrahydro-4-methoxy-6-methyl-1,3-dioxolo[4,5-g]-isoquinolin-5-yl)-1(3H)-isobenzofuranone	413.421	Tas2R14	10
PROP (6-propyl-2-thiouracil)	6-propyl-2-sulfanylidene-1H-pyrimidin-4-one	170.232	Tas2R38	0.11

\*reported in the literature and obtained from BitterDB<sup>80</sup>

## 2.11 APOPTOSIS ASSAY

SKOV3 cells were grown on glass coverslips in 6 well plates and transfected at 50-60% confluency with (-) control siRNA and Tas2R14 siRNA. Cells were then treated with DMSO or 25 $\mu$ M noscapine (MP Biomedicals, Santa Ana, CA) in DMSO for 24 hours. The Annexin V Apoptosis Detection kit (sc-4252 AK, Santa Cruz) was used to determine the rates of apoptosis. Cells were harvested and washed with PBS, then resuspended in Annexin V Assay Buffer following the manufacturer's instructions. Cells were gently shaken in the dark with propidium iodide (PI) and Annexin V-FITC-conjugated stain for 20 min. Cells were then examined by fluorescence microscopy and at least 5 fields of view were recorded using an Olympus IX81 microscope equipped with a Photometrics coolSNAP HQ2 camera and an Excite series 120Q light source. Annexin V stain was excited at 488 nm and images were captured at 525 nm. PI was excited at 535 nm and images captured at 617nm. Rates of early apoptosis were determined by dividing the number of cells that stained positive for Annexin-V divided by the total number of cells<sup>168</sup>.

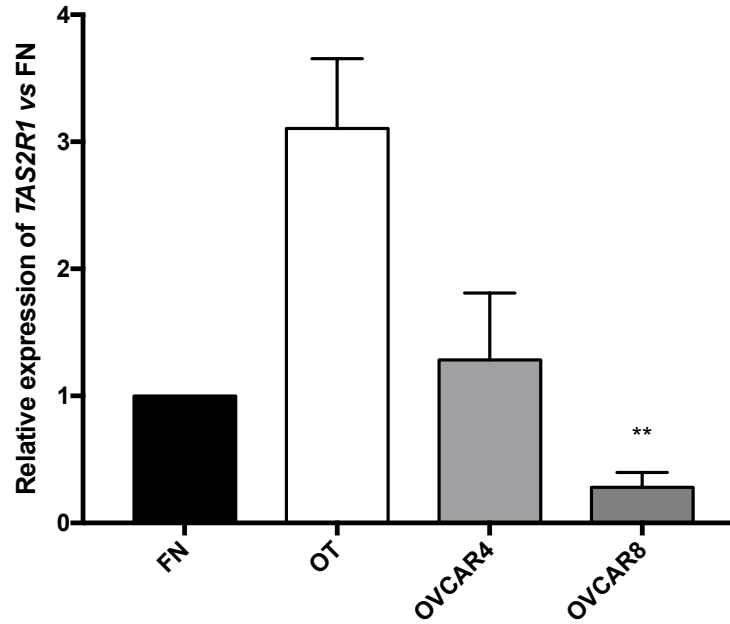
## CHAPTER 3 RESULTS

### 3.1 TAS2R mRNA EXPRESSION IN EOC

qPCR was performed on five candidate TAS2Rs (*TAS2R1*, *TAS2R4*, *TAS2R10*, *TAS2R14*, and *TAS2R38*) using receptor specific Taqman® Probe qPCR probes and cDNA generated from four EOC cell lines of different histotypes including high grade serous (OVCAR4, OVCAR8), clear cell (SKOV3), and the hypermutated IGROV1 cell line originally designated as an endometrioid subtype, as well as RNA from EOC tissue. The  $2^{-\Delta\Delta C_T}$  method was used to assess the relative level of expression of all five TAS2Rs, whereby the difference in threshold cycle ( $\Delta C_T$ ) scores for each gene from the qPCR readout and a reference gene (glyceraldehyde 3-phosphate dehydrogenase, or GAPDH) is assessed for each sample cell line and is compared to the  $\Delta C_T$  of a reference sample ( $\Delta\Delta C_T$ )<sup>166</sup>. As it has become increasingly evident through animal and pathological investigation that high grade serous EOCs may originate from secretory epithelial cells of the distal fallopian tube<sup>169</sup> and that endometrioid and clear cell tumours likely develop from endometrial tissue by retrograde menstruation<sup>170</sup>, the expression of OVCAR4 and OVCAR8 *TAS2R* transcripts was compared to normal fallopian tube mRNA while those of SKOV3 and IGROV1 were compared to normal uterine tissue mRNA. Additionally, *TAS2R* mRNA expression of a uterine/endometrial adenocarcinoma cell line, HEC-1a, was assessed and compared to UN tissue RNAs.

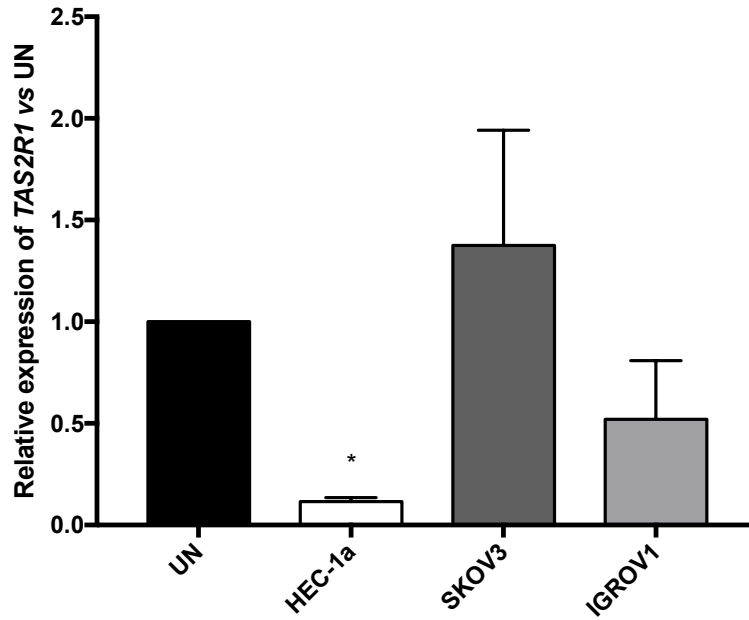
### 3.1.1 Expression of *TAS2R1* mRNA

All the assessed cell lines expressed *TAS2R1* at varying levels; OVCAR8 cells exhibited a significant decrease in expression (72%) of *TAS2R1* mRNA compared to normal fallopian tube tissue (FN), while OVCAR4 cells and ovarian papillary cystadenocarcinoma tissue (OT) expressed 1.3 and 3.1 times as much *TAS2R1* mRNA as FN (Figure 5). *TAS2R1* transcript levels were significantly lower in HEC-1a uterine cancer cells (88.4%) compared to normal uterine (UN) RNA, while those of SKOV3 and IGROV1 did not vary from those of normal uterine tissue (Figure 6).



**Figure 5** **TAS2R1 expression is variable in high grade serous EOC cells.** Results of qPCR analysis comparing the gene expression level of TAS2R1 in high grade serous EOC cells to those of normal fallopian tube tissue (FN). Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta T}$  method with GAPDH as a reference gene. OT: ovarian cystadenocarcinoma tumour tissue RNA.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM. \*\*= $p < 0.01$ .

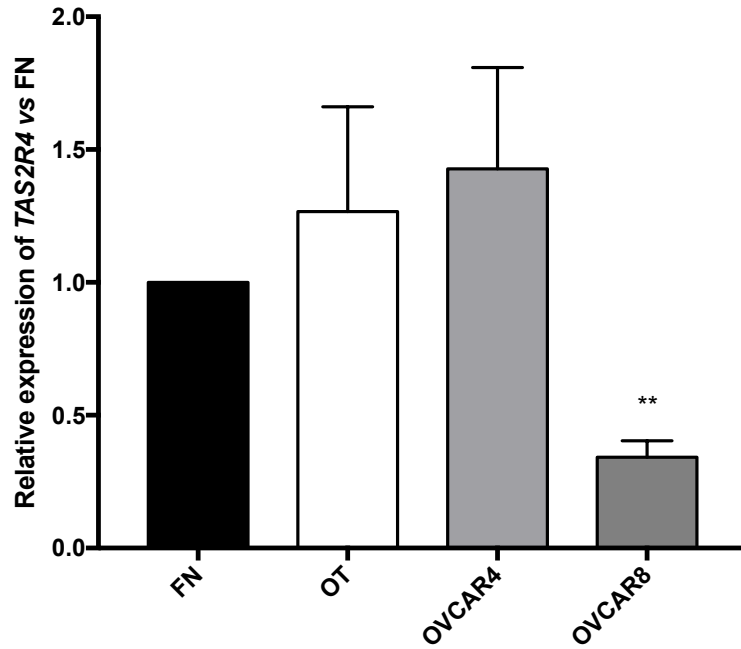




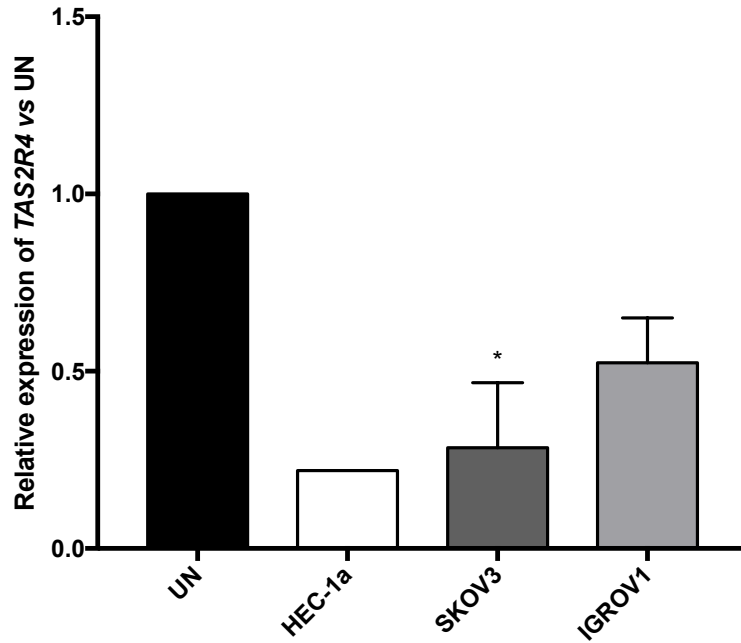
**Figure 6** **Endometrioid-like EOC cell TAS2R1 expression does not vary from baseline.** Results of qPCR analysis comparing the gene expression level of TAS2R1 in endometrioid ovarian cancer cells to those of normal uterine tissue (UN). Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta T}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs UN +/- SEM. \*= $p < .05$ .

### 3.1.2 Expression of *TAS2R4* mRNA

As it has been observed in cells derived from breast tissue that *TAS2R4* mRNA is downregulated in breast cancer cells in comparison to mammary epithelial cells<sup>125</sup>, the expression of *TAS2R4* mRNA was assessed in EOC cells. Compared to fallopian tissue, OT and OVCAR4 cells exhibited non-significant increases in *TAS2R4* mRNA of 27% and 43% respectively, while levels of *TAS2R4* mRNA were 66% lower in *OVCAR8* cells (Figure 7). Additionally, only SKOV3 cells expressed significantly less *TAS2R4* mRNA compared to uterine tissue (72%), while levels of HEC-1a and IGROV1 *TAS2R4* mRNA levels trended downward (Figure 8).



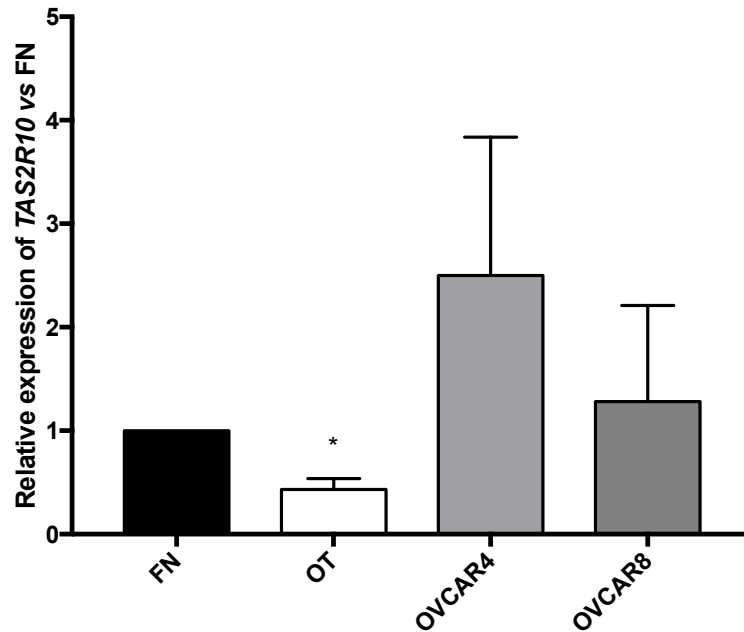
**Figure 7** **TAS2R4 expression is not universally low across HGSEOC.** qPCR analysis of TAS2R4 gene expression in HGSEOC cells compared to normal fallopian tube tissue (FN). Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta CT}$  method with GAPDH as a reference gene. OT: ovarian cystadenocarcinoma tumour tissue RNA.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM. \*\*= $p < 0.01$ .



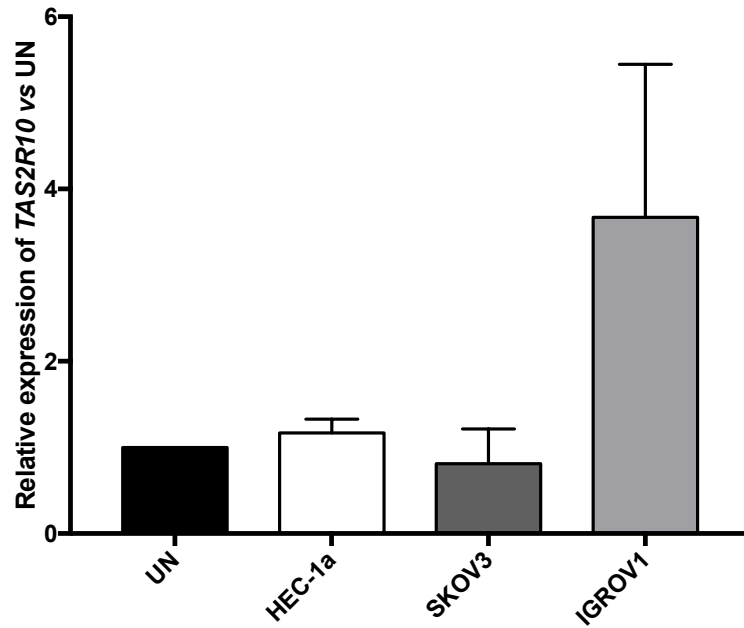
**Figure 8** **TAS2R4 mRNA is downregulated in endometrioid EOCs.** qPCR analysis of TAS2R4 gene expression in endometrioid ovarian cancer cells to those of normal uterine tissue (UN). Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta T}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs UN +/- SEM. \*= $p < .05$ .

### 3.1.3 Expression of *TAS2R10* mRNA

qPCR analysis of *TAS2R10* expression revealed no significant changes in OVCAR4 and OVCAR8 cells compared to FN, but a 56.7% decrease in expression in OT RNA was observed (Figure 9). No significant variation in *TAS2R10* transcript levels were observed for all tested endometrioid-derived EOC cell lines (Figure 10).



**Figure 9** **HGSEOC cells express similar levels of TAS2R10 mRNA as control fallopian tissue.** Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta CT}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM.  $*=p<.05$ .

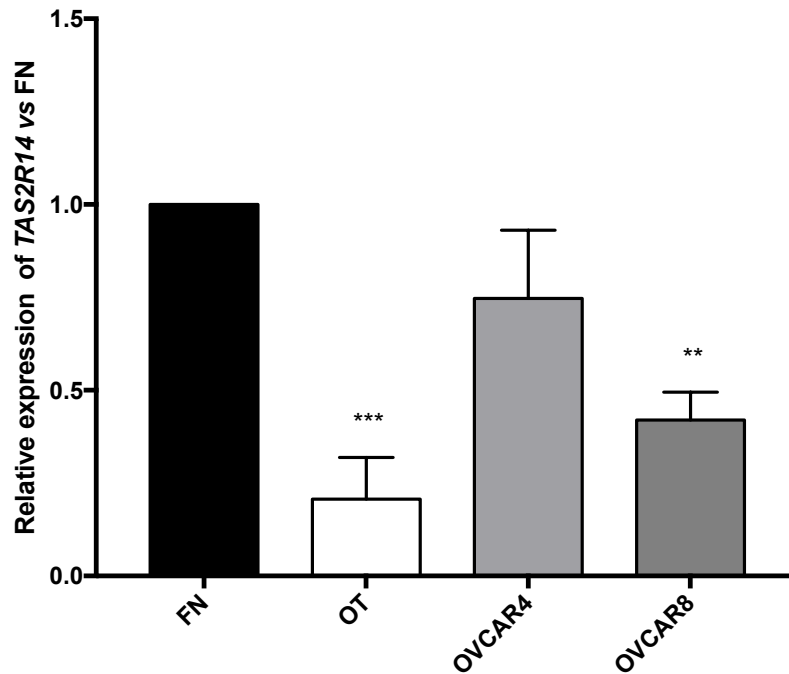


**Figure 10** **TAS2R10 mRNA does not vary significantly in endometrioid EOC relative to healthy uterine tissue.** Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta Ct}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs UN +/- SEM.

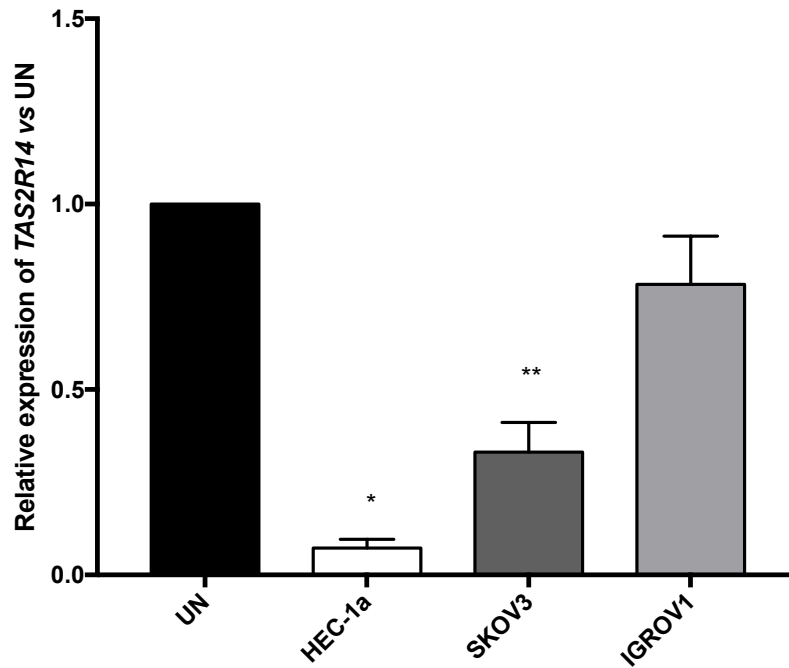
#### 3.1.4 Expression of *TAS2R14* mRNA

Recent studies have identified several anti-proliferative properties of the opium poppy alkaloid noscapine in the treatment of prostate<sup>160</sup>, breast<sup>171</sup> and ovarian<sup>159, 162</sup> cancers. As noscapine is an agonist of the bitter taste receptor Tas2R14<sup>74</sup>, *TAS2R14* transcript levels were assessed in EOC cell lines. Interestingly, the expression of *TAS2R14* mRNA was significantly reduced in HGSEOC, 79% lower in OT and 58% lower in OVCAR8 cells, compared to healthy fallopian tube tissue (Figure 11). *TAS2R14* mRNA levels were also significantly lower in SKOV3 cells and HEC-1a cells (67% and 93% respectively), but not in IGROV1 cells (Figure 12).





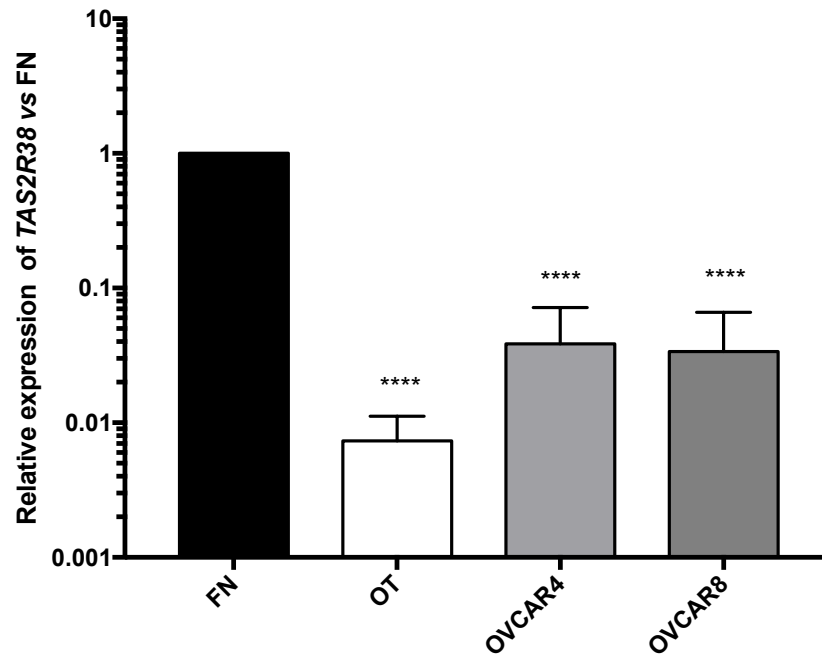
**Figure 11** **TAS2R14 expression is significantly lower in HGSEOC tissue and cells.** Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta T}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM. \*\*= $p < 0.01$ ; \*\*\*= $p < 0.001$ .



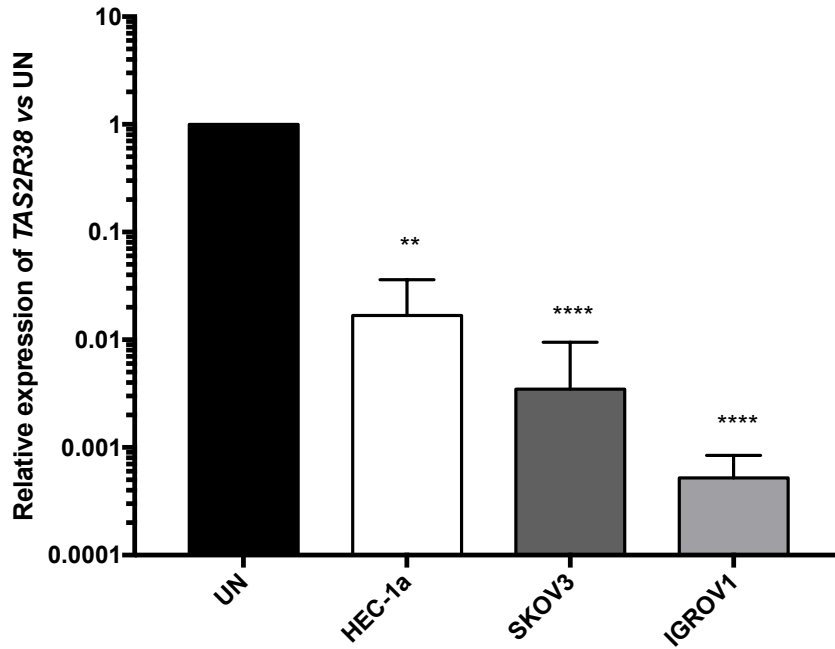
**Figure 12** Endometrioid cancer and endometrioid-like EOC cells express significantly less TAS2R14 mRNA. Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta Ct}$  method with GAPDH as a reference gene.  $n \geq 3$ , two-tailed, paired t-test vs UN +/- SEM. \*= $p < .05$ ; \*\*= $p < 0.01$ .

### 3.1.5 Expression of *TAS2R38* mRNA

The expression of *Tas2R38* and its association with cancer risk has been studied in both colorectal<sup>123</sup> and pancreatic<sup>126</sup> cancers. Because of its possible importance in several different cancers, the expression of *Tas2R38* was characterized in EOC cells and tissues. Transcriptional levels of *TAS2R38* were highly reduced across all HGSEOC and endometrioid-like EOC samples. OT, OVCAR4 and OVCAR8 *TAS2R38* transcript levels were diminished by 99.3%, 96.2% and 96.6%, respectively (Figure 13). Additionally, *TAS2R38* transcription was reduced 98.3%, 99.7% and 99.9% in HEC-1a, SKOV3 and IGROV1 cells, respectively (Figure 14).



**Figure 13** **TAS2R38 transcript levels are highly reduced in HGSEOC.** Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta CT}$  method with GAPDH as a reference gene. Expression is represented on a log<sub>10</sub> scale.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM. \*\*\*\*= $p < 0.0001$ .



**Figure 14** Endometrioid-like EOCs express significantly less TAS2R38 mRNA than uterine tissue. Relative expression for each receptor was determined using the comparative  $2^{-\Delta\Delta Ct}$  method with GAPDH as a reference gene. Expression is represented on a  $\log_{10}$  scale.  $n \geq 3$ , two-tailed, paired t-test vs FN +/- SEM. \*\*= $p < 0.01$ ; \*\*\*\*= $p < 0.0001$ .

### 3.1.6 Summary

To date, there exists little evidence suggesting a strong correlation between mRNA expression levels and those of their corresponding protein products<sup>172, 173</sup>. Despite this, observing changes in mRNA expression profiles remains a reliable technique in diagnosing many diseases such as cancer<sup>174</sup>. As gene expression levels of *TAS2Rs* have been shown to fluctuate in several pathological states<sup>103, 119-121, 125, 175</sup>, the expression of multiple *TAS2R* mRNAs was evaluated in both high and low grade serous EOCs of fallopian and endometrioid origin. While the expression of some *TAS2R* transcripts did not vary from normal healthy tissue, *TAS2R14* and *TAS2R38* mRNA expression was downregulated up to 93% and 99.9% in some EOC cell and tissue subtypes.

**Table 5** Differentially regulated *TAS2R* transcripts in EOC cell lines

Cell line	Subtype (normal tissue)	Expression vs normal tissue				
		TAS2R1	TAS2R4	TAS2R10	TAS2R14	TAS2R38
OVCA4	HGSEOC (FN)					****
OVCA8	HGSEOC (FN)	**	**		**	****
SKOV3	LGSEOC/ Endometrioid (UN)		*		**	****

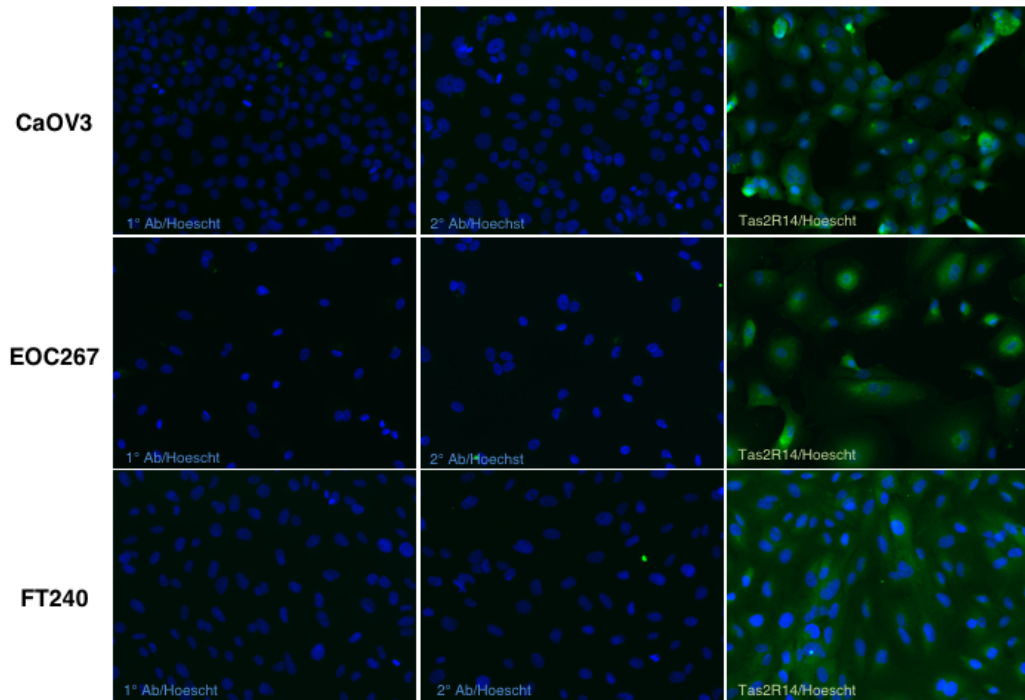
IGROV1	LGSEOC/ Endometrioid (UN)					****
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### 3.2 TAS2R PROTEIN EXPRESSION IN PRIMARY TISSUE SAMPLES

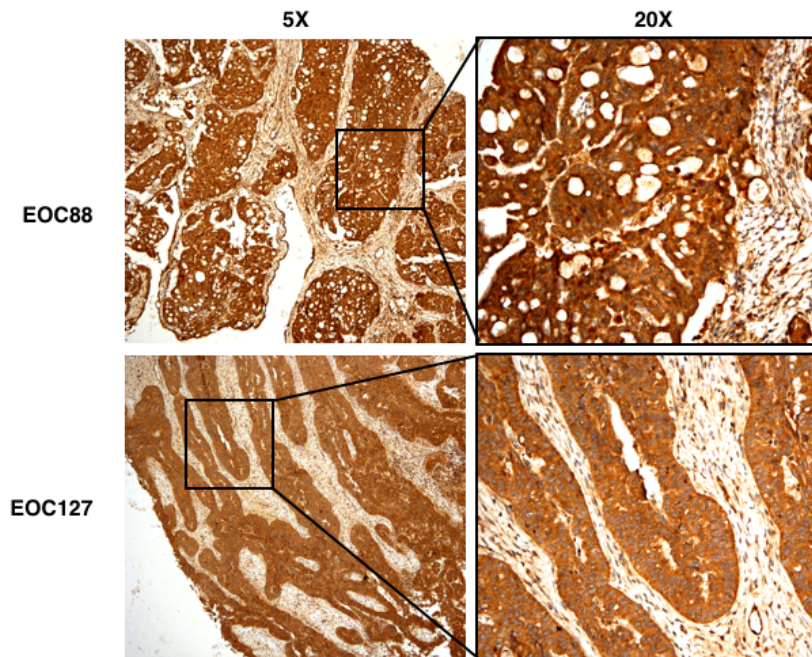
To assess whether *TAS2R* mRNAs are translated and expressed in EOCs, immunological staining for Tas2R14 was performed on several primary HGSEOC cell samples as well as on normal fallopian tube cells to assess protein expression. Immunocytochemical analysis of CaOV3 cells, patient derived EOC cells (EOC267) and fallopian tube cells (FT240) with GFP-tagged secondary antibody revealed that Tas2R14 is expressed in both ovarian cancer and fallopian tube cell types (Figure 15).

To visualize the expression of Tas2R14 with respect to EOC tissue architecture, immunohistochemistry was performed on three HGSOC tissue samples (Figure 16). All samples exhibited staining for Tas2R14 in the epithelial component of the tumour (brown), with notably reduced staining in the stromal component (clear). These results demonstrate that Tas2R14 is expressed at the protein level in EOC cells and tissues.





**Figure 15** **Immunocytochemistry reveals expression of Tas2R14 protein in EOC cells.** Ovarian cancer cells (CaOV3), a primary epithelial ovarian cancer sample (EOC267) and a fallopian tube secretory epithelial cell line (FT240) reveal Tas2R14 protein expression (green). Cells were incubated with primary antibody, secondary antibody or both alongside Hoescht 33342 nuclear stain (blue). Images were obtained and prepared by the Nachtigal lab at the University of Manitoba.

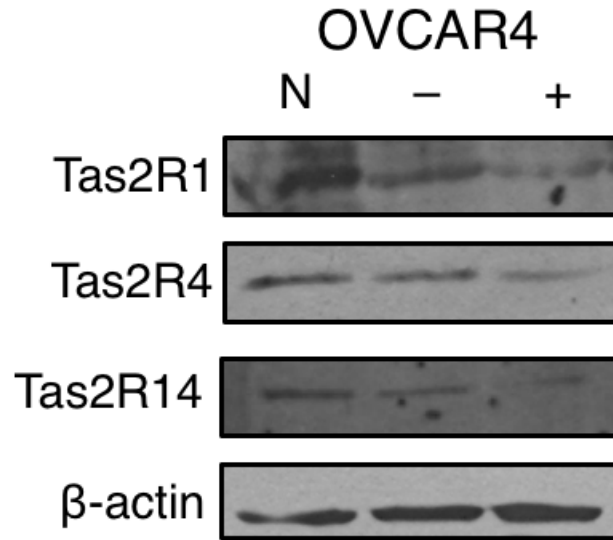


**Figure 16** Immunohistochemistry of two epithelial ovarian cancer primary tissue samples reveals expression of Tas2R14. Images of patient primary tissue samples (EOC88 and EOC127). Both were captured at 5X and 20X (inset). Images were obtained by the Nachtigal lab at the University of Manitoba.

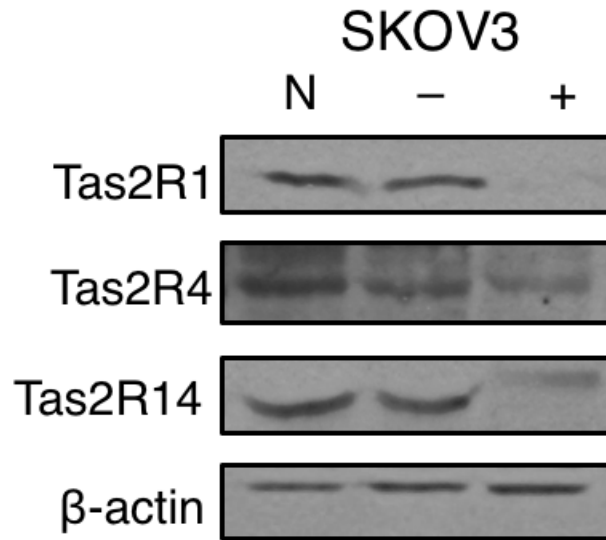
### 3.3 MODULATION OF TAS2R PROTEIN LEVELS USING siRNA

#### 3.3.1 Western blot analysis of Tas2R1, Tas2R4 and Tas2R14

The most common and effective way to elucidate the function of a protein in a tissue or disease context is by impacting its function in order to observe a phenotypic change<sup>176</sup>. Protein function can be influenced by numerous techniques including: introducing loss of function mutations into the protein coding gene of interest, pharmacologically inhibiting expressed proteins or impacting gene transcription at the mRNA level with siRNAs. To determine whether the expression of Tas2R proteins could be modulated, one HGSEOC cell line, OVCAR4 (Figure 17), and one clear cell EOC cell line, SKOV3 (Figure 18), were transfected with a negative control siRNA or pooled Tas2R-specific siRNAs. Cells were harvested 24 h post-transfection and Western blots were performed for Tas2R1, Tas2R4 and Tas2R14 expression. Partial knockdown of Tas2R1, Tas2R4 and Tas2R14 was achieved in OVCAR4 and SKOV3 cells when compared to a  $\beta$ -actin loading control. After the administration of Tas2R-specific siRNAs, protein levels of Tas2R1, Tas2R4 and Tas2R14 were reduced up to 81% in OVCAR4 and SKOV3.



**Figure 17** **Tas2R proteins can be selectively knocked down with receptor specific siRNAs in HGSEOC cells.** Western blot analyses were performed for using rabbit anti-Tas2R14 antibody 24 hours after treating OVCAR4 cells with no siRNA (N), a scrambled control siRNA (-) or a Tas2R14-specific siRNA (+).  $\beta$ -actin was included as a loading control. Western is a representative blot of n = 3 replicates.



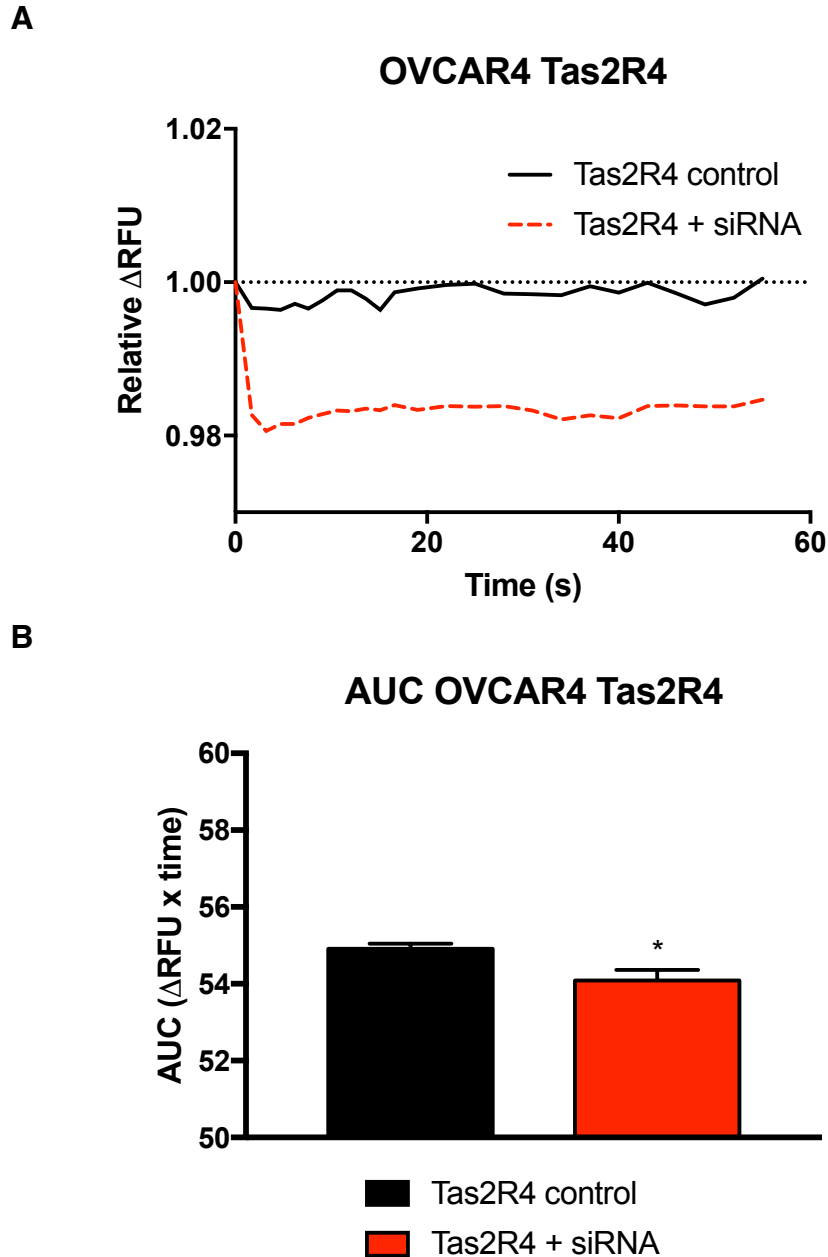
**Figure 18** **Tas2R-specific siRNAs can be used to knockdown Tas2R protein production in clear cell EOC cells.** Western blot analyses were performed for using rabbit anti-Tas2R14 antibody 24 hours after treating SKOV3 cells with no siRNA (N), a scrambled control siRNA (-) or a Tas2R14-specific siRNA (+).  $\beta$ -actin was included as a loading control. Western is a representative blot of n = 3 replicates.

### 3.4 CALCIUM MOBILIZATION ASSAYS

Expression of a protein in a given tissue does not necessarily imply that it is functional in that tissue. As Tas2Rs primarily couple to a  $G\alpha_i$  family G protein,  $G\alpha_{\text{gust}}$ , their stimulation leads to  $IP_3$ -mediated release of intracellular calcium from the ER. Therefore, to assess receptor function through G-protein coupled receptor-mediated calcium release, cells were again transfected with either a negative control siRNA or pooled Tas2R-specific siRNAs and plated onto a 96-well plate for Fluo-4 NW calcium assays carried out 24 hours later. Cells were then stimulated with one of three bitter ligands: colchicine, a toxic natural product from the meadow saffron plant (*Colchicum autumnale*) and Tas2R4 ligand; noscapine, a Tas2R14 agonist from the *Papaver somniferum* poppy; and 6-propyl-2-thiouracil, or PROP, a thioamide drug used clinically to treat hyperthyroidism and selective agonist of Tas2R38. Endogenous calcium release in SKOV3 and OVCAR4 cells was then recorded over 50 seconds following administration of Tas2R ligand. The concentrations of each ligand were determined by literary searches for the threshold concentration (*i.e.* the lowest concentration of test substance capable of producing a physiological response) of each receptor<sup>80</sup>.

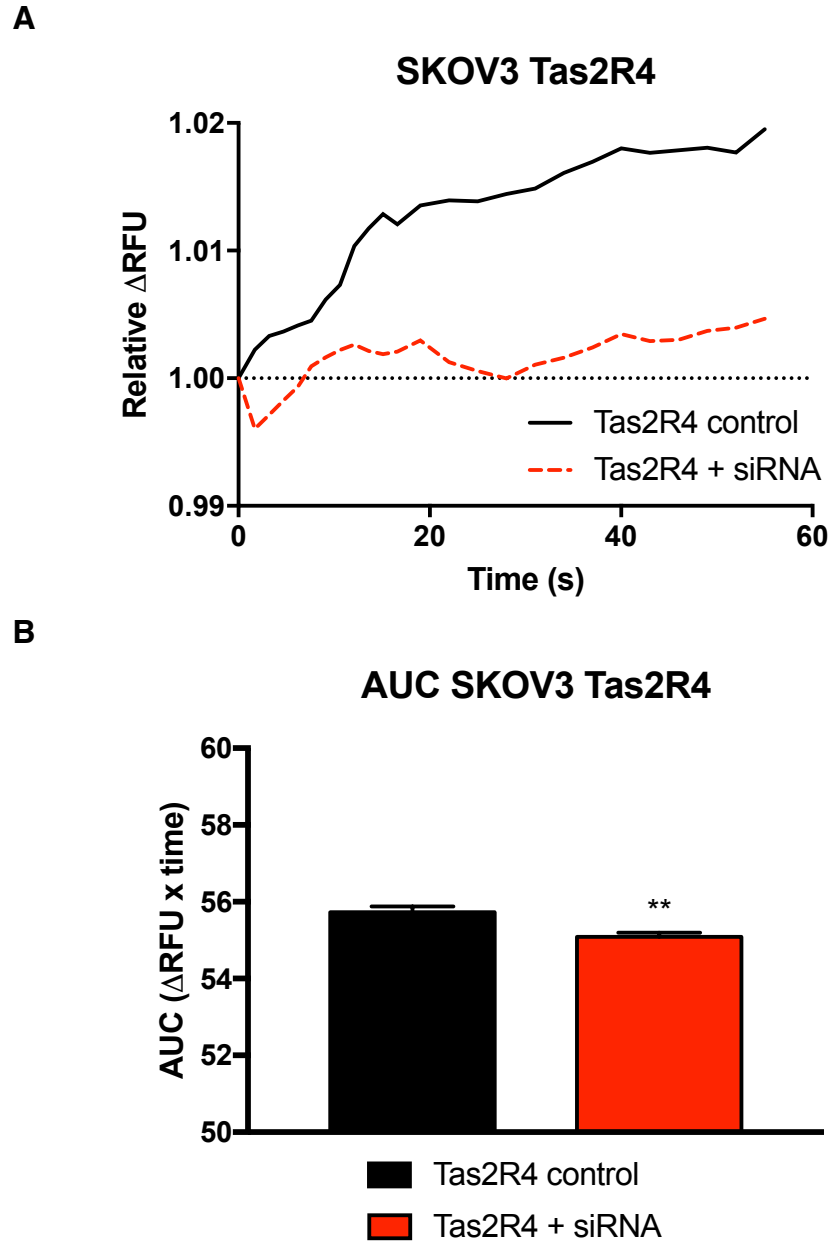
### 3.4.1 $\text{Ca}^{2+}$ release following stimulation of Tas2R4

After administration of 100  $\mu\text{M}$  colchicine, OVCAR4 cells did not appear to experience an increase in calcium release compared to baseline measures of relative fluorescence. Additionally, cells expressing Tas2R4-specific siRNAs appeared to experience a decline in intracellular calcium concentration (Figure 19A). This decline in Fluo-4 bound calcium in Tas2R4-low cells can also be seen when comparing the AUC values of the control siRNA and Tas2R4-specific siRNA calcium traces (Figure 19B). Conversely, stimulating Tas2R4 expressed in SKOV3 cells with colchicine led to modest increases in calcium release, which were abolished significantly upon knockdown of the receptor with siRNA (Figures 20A, 20B), indicating that Tas2R4 is responsive to colchicine stimulation in SKOV3 cells but not in OVCAR4 cells.



**Figure 19** **Colchicine does not induce Tas2R4-mediated calcium release from internal calcium stores.** **A** OVCAR4 cells were treated with control or Tas2R4-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (100  $\mu$ M colchicine). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test.  $*=p<.05$ .



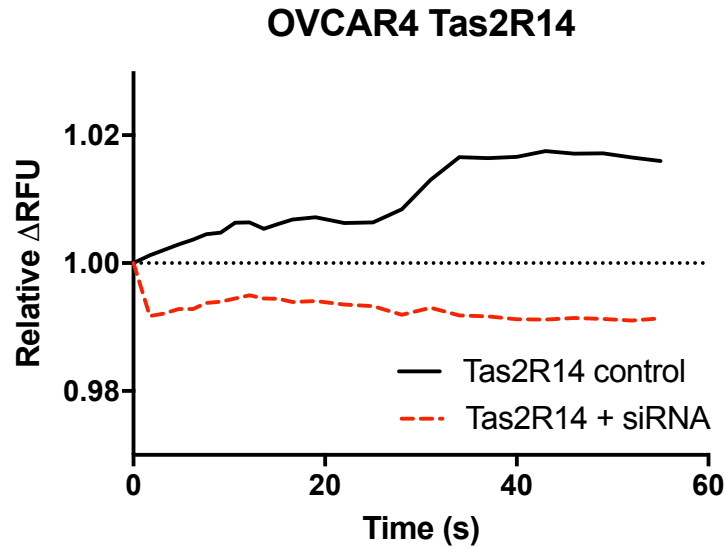


**Figure 20** **Tas2R4 is functional in SKOV3 cells as demonstrated through colchicine-mediated endogenous calcium release.** **A** SKOV3 cells were treated with control or Tas2R4-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (100  $\mu$ M colchicine). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test. \*\*= $p < 0.01$ .

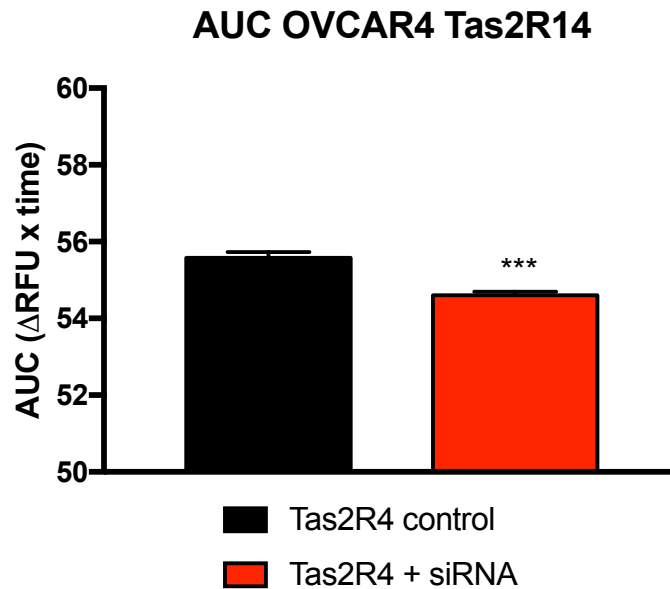
### 3.4.2 $\text{Ca}^{2+}$ release following stimulation of Tas2R14

OVCAR4 cells appeared to express functional Tas2R14 protein, as evidenced by modest increases in calcium mobilization after stimulation with 10  $\mu\text{M}$  noscapine that did not occur in absence of the receptor (Figure 21A). This increase in calcium was significantly larger than observed in Tas2R4-negative OVCAR4 cells (Figure 21B). Endogenous calcium release upon stimulation with noscapine was also observed in SKOV3 cells relative to baseline measurement (Figures 22A, 22B). These results indicate that functional Tas2R14 proteins are expressed on OVCAR4 and SKOV3 cells, and that this effect can be reduced after transfection of cells with Tas2R14-specific siRNAs.

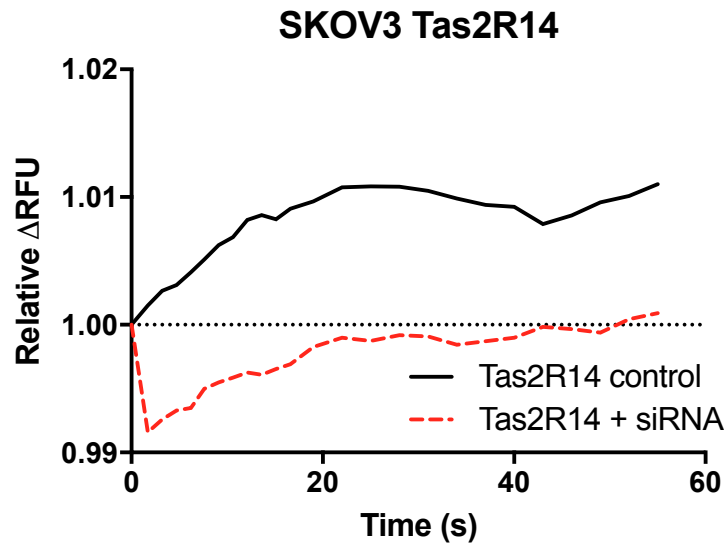
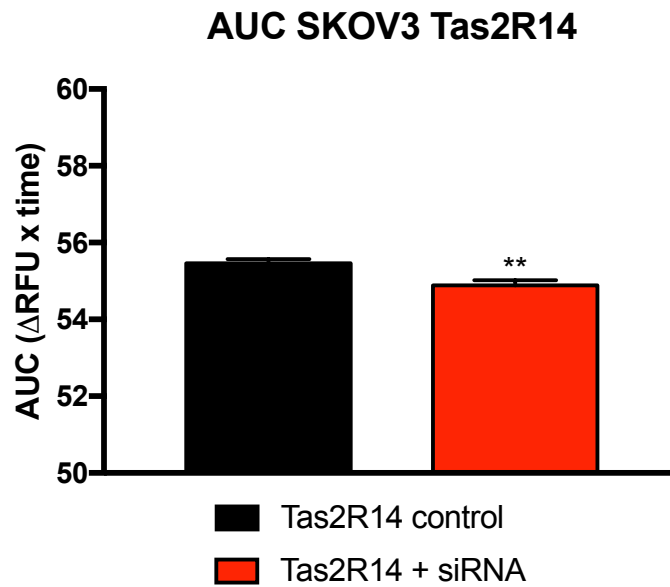
A



B



**Figure 21** HGSEOC cells express functional Tas2R14 as assessed through the monitoring of intracellular calcium mobilization. **A** OVCAR4 cells were treated with control or Tas2R14-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (10  $\mu$ M noscapine). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test. \*\*\*= $p < 0.001$ .

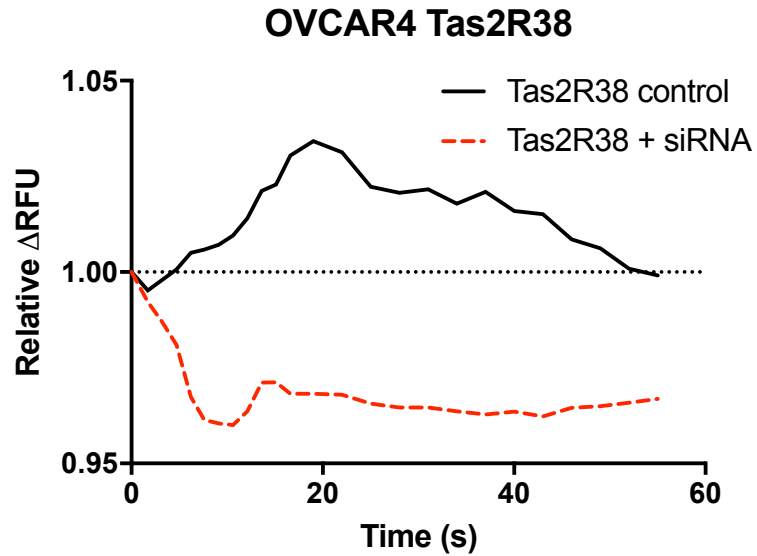
**A****B****Figure 22**

**Noscapine mediated calcium release observed in SKOV3 cells expressing Tas2R14.** **A** SKOV3 cells were treated with control or Tas2R14-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (10  $\mu$ M noscapine). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test. \*\*= $p < 0.01$ .

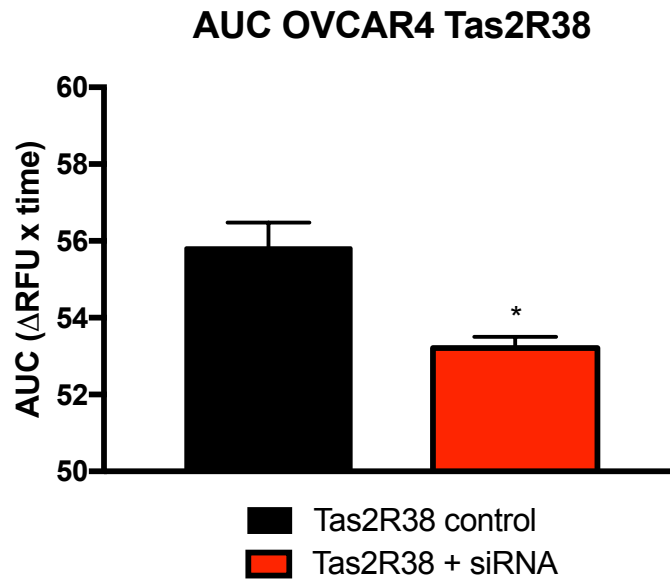
### 3.4.3 $\text{Ca}^{2+}$ release upon stimulation of Tas2R38

The activity of Tas2R38 in OVCAR4 and SKOV3 cells was assessed by monitoring intracellular calcium release as well. As PROP is a Tas2R38-specific ligand and does not activate any other human Tas2Rs, a higher concentration (1  $\mu\text{M}$ ) was used in lieu of its reported effective concentration of 0.1  $\mu\text{M}$ <sup>74</sup> to ensure that activation of the receptor was observable. Stimulation of OVCAR4 cells lead to a robust change in relative fluorescence compared to cells that had been pre-treated with Tas2R38-specific siRNAs (Figure 23A). This increase was significant, as is demonstrated in Figure 22B. However, as in the case of Tas2R4, Tas2R38 activity did not appear to hold over between different EOC subtypes. SKOV3 cells, when stimulated with PROP in the presence and absence of Tas2R38, did not display any increased release of intracellular calcium (Figures 24A, 24B).

A



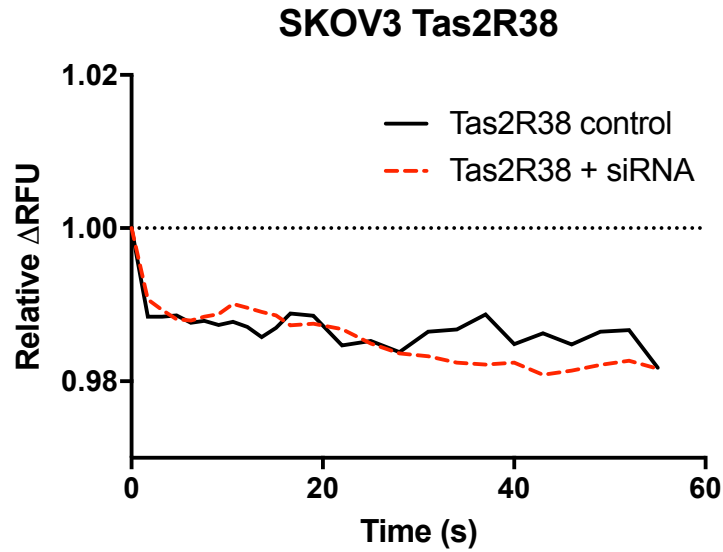
B



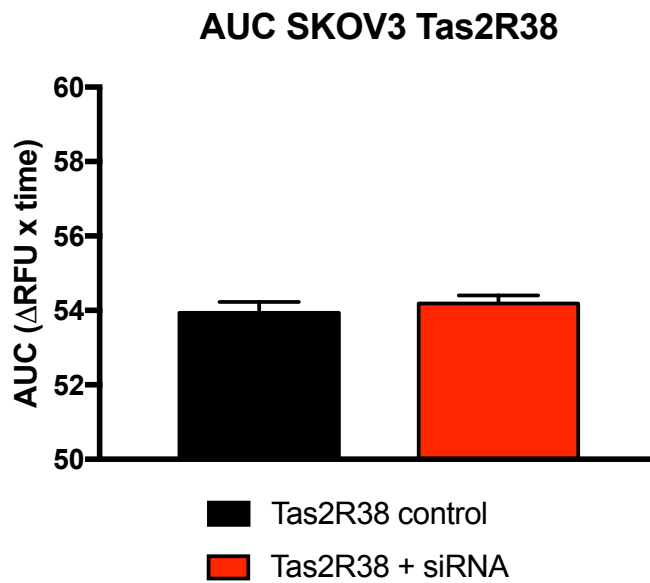
**Figure 23**

**Tas2R38 is functional in HGSEOC cells and stimulation leads to increased calcium mobilization.** **A** OVCAR4 cells were treated with control or Tas2R38-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (1  $\mu$ M PROP). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test.  $*=p<.05$ .

A



B



**Figure 24**

**SKOV3 cells do not express functional Tas2R38.** **A** SKOV3 cells were treated with control or Tas2R38-specific siRNA for 24 hours, and changes in intracellular calcium release was recorded for 50 seconds and traces were plotted as a relative change in fluorescence units ( $\Delta$ RFU) from baseline measurements (dotted black line) before addition of ligand (1  $\mu$ M PROP). Traces are a representative of  $n \geq 5$  replicates. **B** Area under the curve measurements ( $\Delta$ RFU x time) of each representative trace.  $n \geq 3$ , two-tailed, paired t-test.

### 3.4.4 Summary

The expression of four bitter taste receptors was characterized in both a HGSEOC and LGSEOC cell line to determine their role in cellular physiology during ovarian cancer. Their expression in these cells led to the characterization of their signaling ability. Using Fluo-4 calcium imaging following stimulation with Tas2R-specific ligands, the activity of these receptors was assessed by monitoring GPCR-mediated release of intracellular calcium. Both OVCAR4 HGSEOC cells and SKOV3 LGSEOC cells appeared to express functional Tas2R14. Tas2R4 were functionally active in SKOV3 cells only, while Tas2R38 appeared to be non-responsive to PROP stimulation in OVCAR4 cells only, as demonstrated using bitter receptor-specific siRNA-mediated knockdowns (Table 6). These results are similar to those observed in breast cancer cells, in that Tas2R4 was functionally active in MDA-MB-231 and MCF-7 cells after stimulation with a Tas2R4 ligand<sup>125</sup> and to those in pancreatic cancer cells that express functional Tas2R38<sup>126</sup>.

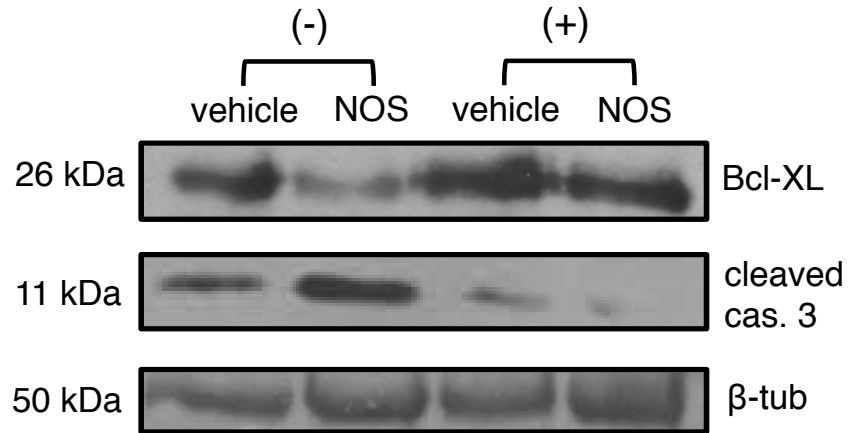
**Table 6 Summary of functionally active Tas2Rs in EOC cells**

<b>Receptor</b>	<b>OVCAR4 (HGSEOC)</b>	<b>SKOV3 (LGSEOC)</b>
Tas2R4	Non-responsive	Functional
Tas2R14	Functional	Functional
Tas2R38	Functional	Non-responsive



### 3.5 WESTERN BLOTTING OF APOPTOTIC PATHWAY PROTEINS

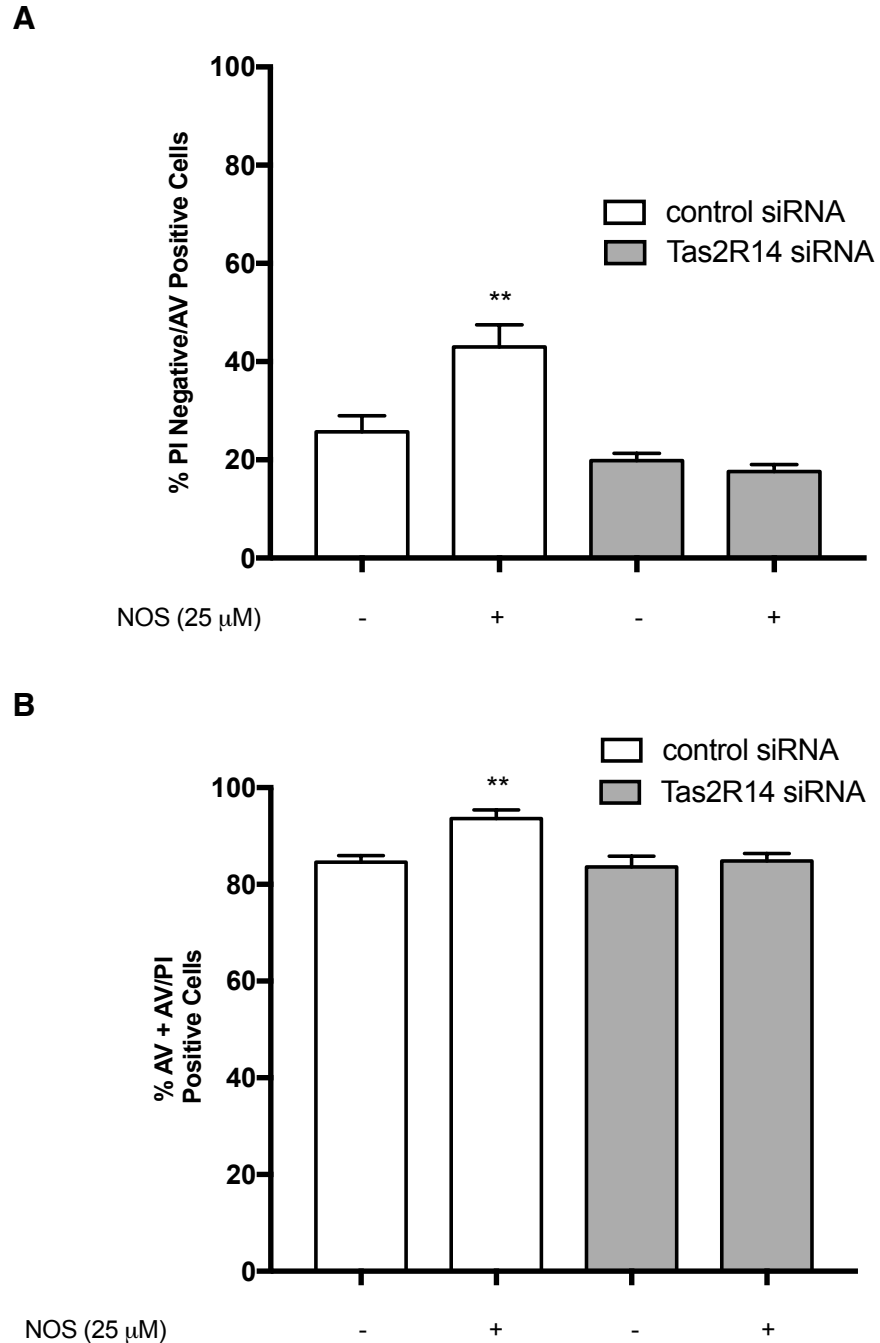
As bitter compounds have been previously identified to promote apoptosis in ovarian cancer<sup>154, 159</sup>, the pro-apoptotic effect of noscapine was assessed in the context of Tas2R14 activation. SKOV3 cells were transfected with negative control siRNA or Tas2R14-specific siRNAs 24 h prior to stimulation with 25  $\mu$ M noscapine for 24 h. The cells were lysed, protein expression was determined by Western blot analysis, and protein expression levels were assessed relative to  $\beta$ -tubulin loading controls. Previously, altered apoptotic protein dynamics have been observed in ovarian cancers after exposure to natural bitter compounds: increases in cleaved caspase-3 among other protein changes, have been observed after exposing EOC cells to noscapine and other bitter compounds<sup>154, 159</sup>. Therefore, the expression of Bcl-XL, a pro-survival protein, and cleaved caspase-3, a pro-apoptotic protein was assessed. Treatment of SKOV3 cells for 24 h with noscapine led to an increase in the expression of cleaved caspase-3 (Figure 25). By contrast, in the presence of Tas2R14 siRNA, the basal level of cleaved caspase-3 were reduced. Additionally, treatment of SKOV3 cells with noscapine for 24 h led to a decrease in Bcl-XL expression, while this effect was reduced when Tas2R14 siRNAs were expressed. Together, these observations implicate the involvement of Tas2R14 in the activation of the apoptotic pathways induced by noscapine.



**Figure 25** **Noscapine induces changes in Bcl-XL expression and caspase-3 activation.** SKOV3 cells were treated with either a negative control siRNA 24 h prior to treatment with 25  $\mu$ M noscapine. Western blotting analysis was then performed on whole-cell lysates using a mouse anti-caspase-3 p11 antibody and rabbit anti-Bcl-XL antibodies.  $\beta$ -tubulin was included as a loading control. Figure is a representative blot of  $n = 3$  experiments.

### 3.6 ANNEXIN V APOPTOSIS ASSAY WITH NOSCAPINE

As exposure to noscapine for 24 hours modulated apoptotic signals, and noscapine has been documented to induce apoptotic cell death in ovarian cancer<sup>159, 177</sup>, an apoptosis assay was performed to determine whether Tas2R14 expression produced noticeable effects on the rates of apoptotic cell death in SKOV3 cells. Annexin V and propidium iodide staining was used on siRNA transfected SKOV3 cells that had been treated with noscapine for 24 hours or vehicle. Cells staining for annexin V (AV) only or both AV and propidium iodide (PI) were counted, and the ratios of AV+/PI- and AV+/PI+ cells were calculated to determine the percentage of cells in the early stages of apoptosis and of cells whose cell membrane has begun to break down, respectively. SKOV3 cells transfected with negative control siRNA and stimulated with NOS for 24 h displayed a 17% increase in AV labelled cells (Figure 26A). This effect was lost upon knockdown of the receptor with Tas2R14-specific siRNAs. Additionally, treatment with noscapine led to a 9.6% increase in cells staining for both AV and PI (Figure 26B).



**Figure 26 Exposure to noscaphine increases rates of SKOV3 cells staining for annexin V and propidium iodide.** Cells were grown on coverslips and transfected with scrambled siRNA (-) or anti-TAS2R14 siRNA (+) for 24 hours, then treated with DMSO or 25μM noscaphine (NOS) for 24 hours. Cells staining for (A) annexin V (AV) only or (B) both propidium iodide and annexin V (PI/AV) were counted from 6 random fields of view on a fluorescence microscope.  $n = 3$ , two-tailed, paired t-test. \*\*= $p < 0.01$ .

## CHAPTER 4 DISCUSSION

### 4.1 GENERAL OVERVIEW

GPCRs make up one of the largest families of signaling proteins in vertebrates, and mediate many diverse signaling cascades. Given that most human tissues express GPCRs on their cell membranes and that they display a wide range of signaling abilities, it is not surprising that GPCR signaling is a major point of consideration in the development of new pharmaceutical strategies. We know that GPCRs can propagate signals by interacting with heterotrimeric G proteins, and that these signaling cascades can impact the transcription of genes associated with proliferation, differentiation, cell survival and cancer<sup>178</sup>. Thus, uncovering the biological implications of functional GPCR expression in a given tissue and characterizing that GPCRs signaling will better help those in the pharmaceutical industry develop new pharmacological strategies for diseases in which few robust treatment options exist.

Despite their obvious promise in the pharmaceutical world, present attempts aimed at identifying robust GPCR-targeting drugs are slow-moving, particularly in cancer. This is due in part to the relative lack of crystal structures available to predict receptor-ligand interactions<sup>179-182</sup>, but also because of industrial practices using high-throughput screen-friendly synthetic chemical libraries and rapid hit-to-lead drug discovery tactics that largely omit the discovery of naturally derived and safer GPCR drugs<sup>183</sup>. Therefore, investigating the potential of GPCRs that have known natural ligands with well-

established risk profiles in tissues of interest can help researchers to identify new therapies for diseases with reduced side effect profiles.

Nearly two decades ago, the Tas2R subfamily of GPCRs was identified as the family of receptors responsible for the transduction of bitter taste stimuli in the oral cavity of vertebrates<sup>43, 44, 54, 184, 185</sup>. It is believed that Tas2Rs originally evolved as a protective mechanism to ingesting toxic bitter-tasting metabolites, and that Tas2R repertoires are impacted primarily by environmental and dietary factors. Some studies have observed a significant correlation between bitter tasting ability and the amount of plant material in an animal's diet<sup>49, 64, 186-188</sup>; however, these studies fail to explain why some non-herbivorous species, such as the coelacanth (*Latimeria chalumnae*) and the domestic cat (*Felis catus*) encode more TAS2R genes than some herbivores. These discrepancies in Tas2R repertoires have been explained away as being impacted by the co-evolution of improved degradation systems in some species<sup>189</sup>, as well as being influenced by the non-strict correlation between toxicity and bitter taste<sup>52</sup>. Additionally, since some bitter tasting phytochemicals exhibit some positive health benefits, it would be expected that active seeking behaviours would alter the gene repertoires of some species<sup>190, 191</sup>. It is, however, the discovery of extra-orally expressed bitter taste receptors that has provided a concrete explanation for why diet does not always negatively or positively influence Tas2R repertoires, and has caused many to reconsider their roles beyond bitter taste<sup>51</sup>.

The first extra-orally expressed bitter taste-recognition molecules were identified in 1996 after several groups observed similarities between enteroendocrine cells of the gastrointestinal tract and taste receptor cells lining the tongue<sup>94, 95</sup>. The activation of these intestinal Tas2Rs was found to impact appetite as well as gastric motility and marked the first time that direct activation of non-oral Tas2Rs had physiological consequences. Another major milestone in the characterization of these receptors came in 2010 when Deshpande *et al.*<sup>116</sup> observed robust Tas2R-dependent relaxation of airway smooth muscle cells upon administration of an aerosolized bitter ligand. Since these initial discoveries, Tas2Rs have been documented in a multitude of non-oral tissues: for example, human, mouse and rat cardiac cells appear to express Tas2Rs, and their expression can be upregulated during periods of starvation<sup>103</sup>; RT-PCR analyses and calcium mobilization assays revealed that functional Tas2Rs are expressed in the brain stem, cerebellum, nucleus accumbens and cerebral cortex<sup>117</sup>; and functional assays have revealed the involvement of Tas2Rs in spermatogenesis and sperm motility<sup>192, 193</sup>. Perhaps the most interesting developments in the identification of extra-orally expressed Tas2Rs however are those involving the deregulation of bitter taste receptors in several human diseases.

As of now, there have been around 15 different disorders in which Tas2Rs are in some way implicated<sup>122</sup>, whether it be through correlative studies or being deregulated in various pathological states. In some tissues, such as the thyroid<sup>119</sup>, heart<sup>194</sup> and oral gingiva<sup>195, 196</sup>, varying stages of disease correlate well with Tas2R SNP haplotype. In others, the expression of some taste receptor mRNA transcripts has been found to be

upregulated (*TAS2R13*, *TAS2R14* and *TAS2R19* in human leukocytes of asthma patients<sup>175</sup>; *TAS2R10* and *TAS2R13* in Parkinson's disease patient brains<sup>121</sup>) or downregulated (*TAS2R5* and *TAS2R50* in Parkinson's disease<sup>121</sup>; *TAS2R4*, *TAS2R5*, *TAS2R14* and *TAS2R50* in schizophrenia patient brains<sup>120</sup>; *TAS2R4* in breast cancer cells<sup>125</sup>). Stimulation of Tas2Rs expressed on lipid droplets of pancreatic ductal adenocarcinoma tumour-derived cells even lead to changes in the expression of active mediators of apoptosis and cell survival<sup>126</sup>, demonstrating the functional consequences of targeting Tas2Rs in diseases such as cancer. As there is an ever-present need to identify new therapeutic drug targets for chronic diseases, further characterization of Tas2Rs in diseased tissues for which few therapeutic strategies exist or that are prone to relapse should be prioritized.

One chronic illness which suffers a relatively low 5-year survival rate due to pathological reoccurrence is ovarian cancer. A lack of robust screening techniques as well as a frequently late stage of presentation of most cases contribute to this statistic; however, resistance to treatment with first-line therapies poses a significant challenge to improving patient outcomes. The standard treatment procedure consists of carboplatin-paclitaxel combination chemotherapy following cytoreductive surgery<sup>197</sup>. Sadly, relapse may occur as early as 4 weeks following combination therapy, leaving clinicians with little to no options for low-toxicity second-line therapies<sup>198</sup>. Thus, the treatment of recurrent and inevitably fatal epithelial ovarian cancers is more palliative in nature, focusing primarily on controlling symptoms and improving the quality of life of patients<sup>199</sup>.



Interestingly, several natural bitter compounds have shown promise as both sensitizing and cytotoxic agents in recalcitrant epithelial ovarian cancers. Extracts from the Chinese bitter melon *Momordica charantia* were shown by Yung *et al.*<sup>154</sup> to inhibit EOC cell growth through AMPK signaling and to improve the efficacy of platinum-based chemotherapies. Additionally, an alkaloid from the opium poppy (*Papaver somniferum*), noscapine, was shown to sensitize paclitaxel-resistant and cisplatin-resistant EOC cells, as well as: induce apoptosis through c-Jun signaling; decrease the expression of the pro-survival factors XIAP, survivin and NF- $\kappa$ B; increase pro-apoptotic caspase-3 expression; inhibit HIF-1 $\alpha$ ; and increase the proportion of G<sub>2</sub>/M cells<sup>159, 162, 177</sup>. Despite these observations, the molecular basis of the perceived benefit of natural bitter products in the treatment of cancer has not been well defined. As noscapine is a ligand for the human bitter taste receptor Tas2R14, we sought to determine whether the anti-tumorigenic effect of noscapine was due to its interaction with ovarian Tas2R14 or by altering microtubule dynamics as it has been previously described to elicit its effects.

#### **4.2 TAS2R MRNA AND PROTEIN IS EXPRESSED IN EPITHELIAL OVARIAN CANCER**

The measure of differentially expressed mRNA transcripts is a rapid and quantitative technique that has been used to diagnose and predict treatment outcomes of many cancers for some time<sup>174</sup>. Since differentially regulated mRNAs better correlate with protein expression levels than non-differentially regulated transcripts<sup>200</sup>, and Tas2R mRNAs appear to be differentially regulated in at least one type of cancer<sup>125</sup>, qPCR analysis was

performed to determine the levels of several *TAS2R* transcripts in several EOC cell lines and patient derived tissue samples (Figures 5-14). At least five bitter taste receptors are expressed in ovarian cancer, and the transcriptional regulation of these receptor genes is variable in high grade serous and low grade serous ovarian cancer cell lines. While the expression of some receptors mRNAs such as *TAS2R14* and *TAS2R38* was repressed in nearly all cell lines observed (Figures 11-14), the expression of others such as *TAS2R4* (Figures 7 & 8) and *TAS2R10* (Figures 9 & 10) were increased compared to control tissue samples. These findings are similar to those documented in breast cancer cells, whereby *Tas2R* expression in breast cancer is altered compared to healthy breast cells<sup>125</sup>. These changes in *TAS2R* mRNA raise several questions with regards to bitter taste receptor expression in tissues and how their expression is controlled. As there are still few published studies indicating the roles of *Tas2Rs* in cancer, further investigation is necessary to determine whether tissues specifically up- or down-regulate the expression of *TAS2R* transcripts and the mechanism of regulation or if other factors participate in the control of *Tas2R* expression in these types of cancer. Typically, changes in expression of mRNAs stem from genomic changes such as mutations, deletions or amplifications in protein coding genes<sup>201</sup>. We therefore performed cross-cancer queries through the cBioPortal for Cancer Genomics (<http://cbioportal.org>) to identify potential changes in copy number alterations (CNAs), mutations and deletions of *TAS2R* genes. Interestingly, 79% of breast cancer patients exhibited increased amplification levels of the 5 *TAS2Rs* investigated in this study, followed by 29% and 24% of prostate and ovarian cancer patients, respectively. These changes in amplification in *TAS2R* genes lend credence to the notion that *Tas2Rs* could be involved in multiple cancers, as genes that are encoded in

amplified (i.e. duplicated) regions of the genome typically correlate well with tumorigenicity<sup>202</sup>.

After characterizing the expression of all five Tas2Rs at the mRNA level, protein expression of three Tas2Rs, Tas2R1, Tas2R4 and Tas2R14 was quantified by Western blot analysis in OVCAR4 (Figure 17) and SKOV3 (Figure 18) cells. Using receptor-specific siRNAs, knockdown of these three Tas2Rs was also observed to varying degrees, demonstrating both the lack of non-specific binding of antibody as well as the ability to modulate the expression of Tas2Rs in EOC cells for functional assays. Additionally, the expression of Tas2R14 in primary epithelial ovarian tumour samples was characterized by immunocytochemistry (ICC, Figure 15) and immunohistochemistry (IHC, Figure 16). ICC revealed expression of Tas2R14 in both EOC and precursor tissue cells of the fallopian tube, and expression of Tas2R14 was demonstrated in several primary epithelial ovarian cancer tissue samples.

These results are intriguing; however, their implications are limited. No analyses of baseline protein levels of Tas2R14 in non-cancerous tissue were conducted, therefore it remains unknown as to whether the expression of Tas2Rs is altered in ovarian cancers compared to physiologically normal ovarian tissue. Similarly, it remains unknown whether the varied levels of mRNA expression of these receptors are due primarily to changes in tissue pathology or factors such as tissue specific promoters, as the levels of some *TAS2R* transcripts seem to change in ovarian cancer cells while others do not change from baseline levels of measurement. Despite this, these results indicate that

Tas2R mRNAs and proteins are expressed in HGSEOCs and LGSEOCs and their expression can be modulated in EOC cell lines with siRNAs.

### **4.3 TAS2R14 IS FUNCTIONAL IN BOTH HGSEOC AND LGSEOC CELLS**

As Tas2R activation leads to increases in intracellular calcium release, the activities of Tas2Rs 4, 14 and 38 were established by conducting calcium mobilization assays by using receptor-specific ligands colchicine, noscapine and PROP<sup>74</sup> over a 50-second time course (Figures 19-24). In OVCAR4 cells, stimulation of Tas2Rs 14 (Figure 21) and 38 (Figure 23) with noscapine and PROP, respectively, lead to modest increases in intracellular calcium, while stimulation of Tas2R4 (Figure 19) with colchicine resulted in no change in calcium mobilization. Interestingly, SKOV3 cells appeared to express functional Tas2R4 (Figure 20) and Tas2R14 (Figure 22) but non-functional Tas2R38 (Figure 24). These results are interesting for two reasons: firstly, they suggest that although HGSEOCs and LGSEOCs may express the same Tas2Rs at relatively similar levels, they are not all functional in both subtypes of EOC. This finding is intriguing as it suggests that the future development of chemotherapeutic agents targeting some Tas2Rs may only benefit a subset of EOC patients, while agents targeting other functional Tas2Rs may have a broader scope of action. Secondly, that cancers in different tissues express different functional Tas2Rs. For example, functional Tas2R38 and Tas2R4 was demonstrated in pancreatic cancer tumour-derived cell lines<sup>126</sup> and breast cancer cells<sup>125</sup>, while in some ovarian cancers Tas2R38 and Tas2R4 stimulation does not elicit changes in intracellular calcium mobilization. Thus, to develop safe therapies aimed at stimulating or inhibiting Tas2Rs, functional analyses of Tas2Rs in tissues other than the target tissue

should be carried out to minimize potential side effects. However, some anomalies in the calcium traces for Tas2Rs 4, 14 and 38 in SKOV3 and OVCAR4 cells should be noted. Most of the compounds that were used during these experiments are not wholly specific to any one Tas2R, which could account for some of the observed increases in intracellular calcium release post-transfection with Tas2R siRNAs. Additionally, due to the relative hydrophobicity of the three ligands used, differences in solubility of these compounds could help to explain the varied degrees of activation (i.e. gradual versus rapid release of intracellular calcium) observed between Tas2R4/Tas2R14 and Tas2R38 (Figures 19-24). Therefore, to accurately determine the activity of these and other Tas2Rs in EOC cells and tissues, additional study of these receptors is necessary to assess whether ligand solubility or co-activated receptors are responsible for the varied levels of activation that were observed during these experiments.

As previously mentioned, several studies have investigated the pro-apoptotic and anti-tumorigenic properties of noscapine in ovarian cancer<sup>159, 161, 162, 203</sup>. As noscapine is an agonist of Tas2R14<sup>74</sup> and our Western blotting and calcium mobilization analyses revealed that functional Tas2R14 protein is expressed in both HGSEOC and LGSEOC cells, we sought to determine whether noscapine's pro-apoptotic effects are dependent on Tas2R14 expression. The functional consequence of noscapine treatment was the induction of significant changes in Bcl-XL and cleaved caspase-3 expression, which was dependent on Tas2R14 protein expression (Figure 25). Despite being a Tas2R14 agonist, noscapine is more commonly known to induce apoptosis by directly binding to tubulin subunits, alter microtubule assembly and induce apoptosis in actively cycling cells<sup>204, 205</sup>.

Our results demonstrate that noscapine's pro-apoptotic effect in this case acts through the activation of an apoptotic pathway dependent on Tas2R14. This effect was demonstrated again by conducting an annexin V apoptotic assay in which exposure to noscapine significantly increased the number of apoptotic cells in EOC cell populations expressing Tas2R14 (Figure 26A). Interestingly, SKOV3 cell populations of all four treatment conditions (*i.e.* negative control siRNA, Tas2R14 siRNA, + noscapine or – noscapine) exhibited high baseline levels of late apoptotic or necrotic cells; in some cases, over 80% of cells that were counted were AV+ or AV+/PI+ (Figure 26B). These levels are higher than basal levels of apoptosis in cancer cells, and could be due to many factors such as cellular stress induced by cell culture in absence of any growth serum, siRNA transfection or inadequate cellular conditions (incubations at room temperature versus 37°C). However, the observed increase in cells that were early apoptotic (AV+) demonstrates that noscapine's pro-apoptotic effect is indeed mediated through Tas2R14 in EOC cells.

The observation that noscapine may induce apoptosis through the activation of Tas2R14 is novel. As noscapine has already been used in clinical trials for several different cancers<sup>206, 207</sup>, it is important to determine all of noscapine's potential methods of action to reduce off target effects and to minimize toxicity. Noscapine's role in altering microtubule assembly dynamics has been well established<sup>177, 207, 208</sup>, although the mechanism by which noscapine enters the cell to affect microtubule dynamics has yet to be discovered. Interestingly, Tas2R14 protein expression was observed not only on at the cell membrane, but in the cytosolic compartment of CaOV3 and patient derived EOC

cells as well (Figure 15). It is possible that this increased cytosolic expression of Tas2R14 could be explained as being a product of cellular internalization of membrane-bound Tas2R14, and that upon binding to cell-surface Tas2R14 noscapine is internalized through an endocytic process.

#### **4.4 DOES TAS2R14 SIGNAL THROUGH RAC-1 IN EOC?**

As previously mentioned, Tas2Rs signal in a  $G\alpha_{i/o}$ -dependent manner through association with the G proteins  $G\alpha_{\text{gust}}$ ,  $G\beta 3$  and  $G\gamma 13$ . Upon activation and binding of a bitter ligand,  $G\alpha_{\text{gust}}$  inhibits the conversion of ATP to cAMP by inhibiting AC and the  $G\beta\gamma$  heterodimer activates PLC. PLC converts  $\text{PIP}_2$  to DAG and  $\text{IP}_3$ , the latter of which binds to  $\text{IP}_3$  receptors on the smooth ER and stimulates the release of intracellular calcium. In the oral cavity, rises in intracellular calcium activate TRPM5 channels, leading to cellular depolarization and release of ATP from TRCs. However, the role of Tas2R-mediated calcium release in activating intracellular signaling cascades or other components of the bitter taste cascade as secondary messengers is not as well understood. To date, only several extra-oral signaling mechanisms of Tas2Rs have been proposed: on airway smooth muscle cells and tissue<sup>116, 209</sup>; on ciliated cells of the airway epithelium<sup>210-212</sup>; on enteroendocrine cells of the gut epithelium<sup>104, 213</sup>; in the vomeronasal organ in rats and human SCCs in the nasal canal<sup>107, 113, 114, 214, 215</sup>; on urethral brush cells<sup>216</sup>; and tuft cells of the gut related to immunity from parasitic infection<sup>217-219</sup>. Unfortunately, only the effects of bitter ligand stimulation of tissues on paracrine signaling have been observed, and until recently there had been no documented examples of Tas2R signaling regulating mitogenic or anti-proliferative signaling cascades. Additionally, the molecular basis

behind the observed effects of bitter extracts and compounds on cancer cells has not been well described. Our findings in EOC suggest that Tas2R14 mediates the bitter ligand-induced anti-tumorigenic effect of noscapine. Although promising, further experiments will be needed to completely explain the series of events that eventually lead to the activation of caspase-3 and apoptosis in EOC cells following treatment with noscapine.

Recently, Sidhu *et al.* demonstrated in a Tas2R4/ $G\alpha_{\text{gust}}$ -expressing model that stimulating Tas2R4 could affect the activity of the Rac-1 GTPase<sup>220</sup>. Rac-1 (Ras-related C3 botulinum toxin substrate 1), which is a Rho-family GTPase that is activated by a wide range of receptor tyrosine kinases and GPCRs<sup>221</sup>, plays a role in regulating the cell cycle and has been associated to influence tumorigenesis and metastasis in its active form<sup>222-225</sup>. Interestingly, HEK293T cells treated with quinine, a Tas2R4 agonist, significantly reduced the activity of Rac-1<sup>220</sup>. This effect was reversed by using an inverse agonist of Tas2R4,  $N\alpha,N\alpha$ -bis(carboxymethyl)-L-lysine, or BCML. Additionally, they found that intracellular calcium only partially impacts Rac-1 activity after pre-treating quinine-treated cells with a  $Ca^{2+}$  chelator, indicating an additional yet to be discovered pathway, possibly through the activation of Rac1-specific GAPs influencing the levels of active Rac-1. These findings are intriguing to our study of Tas2Rs in EOC, as they provide a potential connection to the activation of caspase-3 and induction of apoptosis. Active Rac-1 has been shown to promote cell survival in several cell models<sup>226-229</sup>, and a reduction in the GTP-bound form of Rac-1 has been shown to increase the activation of pro-apoptotic ERK signaling via a PI3K/Akt/ MEK pathway independent of Ras<sup>230</sup>. Additionally, active Rac-1 was shown to increase the amount of Bad phosphorylation and



lead to a decrease in pharmacologically induced caspase-mediated apoptosis<sup>231</sup>. Thus, monitoring the levels of GTP-bound Rac-1 after stimulation with noscapine or other Tas2R14 agonists could provide insight into the entire mechanism of Tas2R14-induced apoptosis in epithelial ovarian cancer. One way in which to monitor whether the Rac-1 GTPase is involved in Tas2R14 signaling would be to observe whether Tas2R activation presented any effects on cellular morphology. As Rac-1 knockouts in mouse embryonic fibroblasts have been shown to have reduced organization of the actin cytoskeleton and lowered focal adhesion<sup>232</sup>, it would be expected that noscapine would impact cytoskeletal organization if Rac-1 were implicated in the pathway downstream of Tas2R14.

#### **4.5 FUTURE WORK**

Much more work is necessary to fully characterize the intracellular signaling of Tas2R14 and the long-term implications of its activity in EOC. The realization that Tas2Rs may act to inhibit Rho family GTPases<sup>220</sup> is interesting, and measuring the amount of active Rac-1, ERK, PI3K and AKT could help us in elucidating the entirety of the Tas2R14 apoptotic cascade induced by noscapine. Including other pro-survival and pro-apoptotic proteins such as XIAP, survivin, APAF-1 and NF- $\kappa$ B would help to provide a better picture of the overall biology of EOC Tas2R14 activation, as changes in these proteins were observed by Shen *et al.*<sup>159</sup> after treating platinum-resistant ovarian cancer cells with noscapine. As previously mentioned, many studies have used natural bitter compounds such as noscapine to treat cancers and have observed changes in the proportions of G<sub>1</sub> and G<sub>2</sub>/M cells<sup>159, 161, 162, 203</sup>. Therefore, it would be interesting to perform FACs analysis

on EOC cells expressing and lacking Tas2R14 to determine whether noscapine induces receptor-specific changes in the number of cells at various stages of the cell cycle.

Another interesting avenue in which to continue these studies would be to investigate the dietary regulation of Tas2R mRNAs and proteins in EOC. Previous studies concerning Tas2Rs in the heart<sup>103</sup> have demonstrated that forced starvation and re-implementation of bitter compound-rich diets in mice influence changes in Tas2R mRNA and protein expression. Thus, cell and *in vivo* models that monitored the changes in the expression of ovarian Tas2Rs after sustained high or low bitter diet, or treatment with Tas2R ligands such as noscapine, would provide insight into whether Tas2Rs are viable and robust chemotherapeutic targets for EOC. Additionally, there has been a growing interest in identifying endogenous bitter ligands that could influence extra-oral Tas2R activation such as hormones and amino acids<sup>51</sup>. As the ovaries, much like the heart, are outside of the gastrointestinal tract and do not interact directly with bitter compounds in food, the changes in their expression could be attributed to hormonal influences. Thus, characterizing the molecules involved in internal Tas2R regulation could better help us identify novel therapeutic approaches in ovarian and other cancers.

#### **4.6 CONCLUSIONS**

It has become increasingly difficult to consider Tas2Rs as merely the receptors responsible for the perception of bitter taste, as the body of evidence suggesting their roles beyond the oral cavity is ever-growing. In this study, we show that bitter taste receptor mRNAs and proteins are expressed in ovarian carcinomas, and that the

activation of Tas2R14 with a bitter ligand affects cell survival. Our results identify another type of cancer, after colorectal<sup>123</sup>, breast<sup>125</sup> and pancreatic cancers<sup>126</sup>, to express functional bitter taste receptors. Our findings are preliminary, and demonstrate that noscapine's pro-apoptotic effect in epithelial ovarian cancer cells is mediated through a receptor-mediated process, likely in addition to its effect on microtubule dynamics. To fully understand whether Tas2Rs present a possible new target in EOC and other cancers, further investigation of this receptor family's intracellular signaling pathways is necessary to aid in the discovery of new and potentially safer therapeutic strategies for cancer patients.

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