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(Review)

Unanswered Questions Regarding Sex and BMP/TGF-β signaling

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Abstract: Crosstalk between the BMP and TGF-β signaling pathways regulates many complex developmental processes from the earliest stages of embryogenesis throughout adult life. In many situations, the two signaling pathways act reciprocally. For example, TGF-β signaling is generally pro-fibrotic whereas BMP signaling is anti-fibrotic and pro-calcific. Sex-specific differences occur in many diseases including cardiovascular pathologies. Differing ratios of fibrosis and calcification in stenotic valves suggests that BMP/TGF-β signaling may vary in men and women. In this review, we focus on the current understanding of the interplay between sex and BMP/TGF-β signaling and pose several unanswered questions.

Keywords: BMP, TGF-β, signaling, sex, chromosomes, XIST, genomic imprinting, hormones, fibrosis

1. Introduction

The distinct developmental mechanisms that bring about the dramatic differences in male and female characteristics are well studied. However, the impact of sex-associated signaling on the bone morphogenetic protein (BMP), transforming growth factor (TGF)-β, and other pathways in developing animals and during the adult life is incomplete. Cardiac valvulogenesis is just one of the many complex developmental processes where both BMP and TGF-β signals – along with WNT, fibroblast growth factor (FGF), NOTCH, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) signals - orchestrate differentiation and morphology [1-3]. As in many adult diseases, processes that control normal embryonic processes also are involved in valve pathologies. Recent reports described dissimilarities in the ratios of fibrotic to calcified tissue in equally stenotic valves from men *versus* women [4-7]. Because TGF-β signaling is generally pro-fibrotic and BMP signaling is generally anti-fibrotic, we wondered if sex-associated changes in this balance contributed to the skewed sex distribution of valvular heart disease [4,7-11]. Here we review the current state of understanding regarding the impact of sex on BMP and TGF-β signaling and identify several unanswered questions.

2. Sex Chromosomes. Most biological discussions of sex start with the X- and Y-chromosomes. Basic mechanisms that may influence BMP and TGF-β signaling would include X- or Y-linked inheritance of variant alleles and differences in X-chromosome inactivation. Of 64 mammalian BMP/TGF-β ligands, receptors, canonical signal mediators, and extracellular and intracellular antagonists, only *Bmp15* (GDF9B) maps to the X chromosome (Supplemental Table 1 and [12-15]). BMP15, an oocyte-derived growth and differentiation factor, is essential for folliculogenesis and granulosa cell function and thus female fertility [16]. Of greater overall impact to female biology, both the BMP and TGF-β signaling pathways regulate a key factor in X-chromosome inactivation. This critical process assures similar ratios of X to autosome gene expression between XY males and XX females. In female cells, one of the two X chromosomes is transcriptionally inactivated by a mechanism involving the

noncoding RNA XIST. Six members of the BMP/TGF-β signaling pathway were identified in a screen for X-chromosome inactivation modulators [17]. Chromatin immunoprecipitation experiments determined that BMP signaling directly induced the expression of XIST. In contrast, TGF-β1 down-regulated XIST *in vitro* and *in vivo*. Antagonism between these two pathways would profoundly influence X-chromosome dosage compensation and thus female biology on a cellular level [17].

Unanswered questions. Human X-chromosome inactivation and reactivation have profound consequences on cellular reprogramming and disease [18]. Likewise, BMP/TGF-β signaling strongly impacts pluripotency and differentiation. How does the upstream modulation of XIST expression by BMP/TGF-β signaling impact the downstream processes affected by these pathways? Does the ratio of BMP/TGF-β signaling affect the 15% of X-linked genes that escape from X-inactivation in human females, many in a tissue-specific pattern [19]? What is the relationship between pathological conditions that alter X-chromosome inactivation and BMP/TGF-β signaling?

3. Genetic imprinting is another influential cellular process controlled by sex, in this case, that of the parents. Imprinted genes are expressed on either the maternal or paternal allele, but not both. This monoallelic expression causes a variant or mutated allele to produce a different phenotype based on the parental origin of that imprinted gene. 250 human and 150 mouse imprinted genes were surveyed (Supplemental Table 1 and [20,21]). The ligand, BMP8B, is predicted to have a paternal genetic imprint in humans. The extracellular antagonist, Decorin, is maternally imprinted in mouse, but not humans [21]. Furthermore, at least two imprinted long noncoding (lnc) RNAs (H19 and MEG3) and their miRNA derivatives (*e.g.*, miR-675-3p and -5p) have been shown to regulate key BMP/TGF-β ligands and signaling intermediaries, including BMP4, SMAD1, and SMAD5 [22,23]. These two lncRNAs are imprinted in both humans and mice and have been shown to influence mesenchymal stem cell lineage decisions such as myogenesis, adipogenesis, and osteogenesis that BMP/TGF-β also direct.

Unanswered questions. The epigenetic regulation of imprinted genes controls fetal and postnatal growth, with lifelong metabolic consequences such as obesity that impact health [24]. BMP and TGF-β signaling govern the differentiation of cells into myoblasts, adipocytes, chondrocytes, or osteoblasts. How do parentally imprinted regulators impact critical differentiation choices controlled by BMP/TGF-β signaling?

4. Hormones. Beyond the cell-intrinsic impact of the sex chromosomes and imprinting, hormones such as estrogen and androgens are essential drivers of female and male characteristics and function. Furthermore, the natural developmental variation in hormonal milieu, for example during puberty, pregnancy, lactation, and menopause, is substantial. In contrast to X-chromosome inactivation and imprinting which alter the intrinsic nature of each cell, hormones are extrinsic factors that coordinate cell behaviors on a physiological scale. Unsurprisingly, sex hormones directly regulate many members of the BMP/TGF-β signaling pathways (Table 1). For example, estrogen directly induces *Bmp2* and *Bmp6* transcription [25,26]. In contrast, estrogen inhibits TGF-β signaling by stimulating SMAD2/3 protein degradation [27]. Testosterone was shown to significantly alter the expression of 20 members of the BMP/TGF-β pathway in skeletal muscle progenitors (satellite cells, [28]). Perhaps

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fueled by the long history of reproductive endocrinology - anti-Müllerian hormone (AMH) also known as the Müllerian-inhibiting substance (MIS) was discovered in 1947 [29] – an exceedingly complex network of estrogen and androgen interactions with BMP/TGF-β pathways has been described in reproductive organs. Estrogen and androgens interact with nearly all the members of the TGF-β superfamily (TGF-βs, BMPs, activin, inhibins, anti-Müllerian hormone, growth differentiation factors (GDFs), LEFTY, and NODAL) in females [16]. The system of BMP/TGF-β signaling may be only slightly less vast in males [30,31].

Table 1: BMP/TGF-β signaling pathway members with molecular evidence of direct regulation by sex-related steroids.

Protein	Effector	Evidence	Cell or Tissue type	Reference
Ligands				
AMH (MIS)	Estrogen	Luciferase reporter assay	KK1 cells	[20]
BMP2	Estrogen	Luciferase reporter assay, ovariectomy	C3H10T1/2 cells, bone marrow mesenchymal stem cells	[25,32]
BMP6	Estrogen	Luciferase reporter assay	MCF-7, T47-D cells, and HepG2 cells	[26]
INHβA (ACTA)	Estrogen	Luciferase reporter assay	GRMO2 granulosa cells	[33]
INHβB (ACTB)	Estrogen	Luciferase reporter assay	GRMO2 granulosa cells	[33]
TGF-β1	Dihydrotestosterone, R1881 synthetic androgen	Luciferase reporter assay, Chromatin Immunoprecipitation	PC3mm2 cells, LNCaP cells, primary osteoblasts	[34-36]
TGF-β3	Estrogen	Chloramphenicol Acetyl Transferase (CAT) reporter assay	Human MG63 osteosarcoma cells	[37]
Extracellular Inhibitors				
Decorin	Progesterone, Dienogest synthetic progestin	Chromatin Immunoprecipitation	EMOsis cc/TERT and	[38]

			CRL-4003 cells.	
Receptors				
TGFβR1 (ALK5)	Estrogen	Luciferase reporter assay	osteoblasts	[39]
Intracellular Signal Transducers				
SMAD3	Dihydrotestosterone	Luciferase reporter assay	prostate cancer cell lines	[40]
Intracellular Inhibitors				
SMURF1	Mibolerone synthetic androgen	Chromatin Immunoprecipitation	LNCaP cells	[41]

Unanswered questions. A daunting web of BMP/TGF-β additive, synergistic, and antagonistic actions among members of the ligand superfamily and signal mediators occurs in reproductive tissues. Each interaction within this panapoly "may" occur in other tissues. The challenge is to identify which interactions also occur in other tissues.

5. Crosstalk and balance. In just the context of cardiovascular biology, sex profoundly influences heart and vascular health with estrogen generally playing a protective role [42,43]. That a "one size fits all" approach to cardiovascular treatment cannot work for men and women is now widely recognized [43]. Several cardiovascular diseases, including aortic valve stenosis, exhibit sex-specific differential levels of calcified and fibrotic tissues. The reduced blood flow associated with aortic valve stenosis is life-threatening. Unfortunately, treatment is limited to surgical replacement. Both calcification and fibrosis impair valve leaflet mobility. Studies have shown that valves from men typically have greater aortic valve calcification whereas women have a greater fibrosis score despite equal levels of stenosis and loss of function [5-7]. We postulate that the balance of BMP/TGF-β signaling may differ in the valves from males and females. Many members of the TGF-β superfamily, particularly the founding members TGF-β1 and -β2, but also activin A, myostatin, and BMP9, promote fibrosis in various tissues [4,44]. Others, for example, TGF-β3, BMP2, and BMP7 oppose fibrosis in multiple organs [44-54]. Different ligands often promote alternative lineage choices. For example, TGF-β1 inhibits calcific nodule formation in aortic valves *in vivo* by inducing SOX9, a pro-chondrogenic, anti-osteogenic transcription factor [55]. On the other hand, BMP2 and its downstream effectors, *e.g.*, phosphorylated SMAD1/5/8(9), are potent pro-osteogenic signals strongly implicated in pathological calcification [56-61]. In a few models of organ fibrosis, the antagonistic nature of TGF-β and BMP signaling has been directly observed in the same tissues. In these *in vitro* and *in vivo* studies, TGF-β signaling promoted extracellular matrix synthesis and epithelial-mesenchymal transition (EMT), whereas BMP2 negatively regulated these pro-fibrotic processes [45,62,63]. Understanding this interplay between BMP and TGF-β signaling will potentially reveal potential strategies to control fibrotic, calcific, and other pathologies [44,64].

Unanswered questions. Clear sex-specific differences in the relative levels of calcification and fibrosis in aortic valves occur. Although BMP/TGF-β signaling strongly influences these processes,

few studies have addressed the regulation of these pathways in each sex. What are the relative levels of each BMP/TGF-β ligand in healthy and diseased valves in men and woman? How do sex and hormonal status influence the relative activities of signaling mediators and extracellular and intracellular antagonists of signaling? Most importantly, what therapeutic strategies may modulate the balance of BMP/TGF-β signaling optimally for each sex?

6. Concluding remarks. Many factors lead to sex-specific differences in disease incidences and manifestations and to therapeutic efficacy [65]. These include cell-intrinsic genetic and cell-extrinsic physiological dissimilarities as well as environmental circumstances such as healthcare inequities [66]. Although increased attention is now paid to social and organismal contrasts between males and females, far less is known regarding the impact of sex on biochemical signaling mechanisms. Despite extensive differences in many diseases, preclinical studies often ignore sex as an important biological variable. Studies often use only male animals or fail to report sex at all. Full understanding of disease processes will only be possible when the effect of sex on signal crosstalk is elucidated. The potential reward will be therapeutic methods to fine-tune the balance of networks involving BMPs, TGF-βs, and other signals in both men and women.

Supplemental Table 1: Chromosomal locations and genomic imprinting status for members of the BMP/TGF-β signaling pathways.

Protein	HNGC ID	Chromo-some (human/ mouse)	Imprinted	Protein	HNGC ID	Chromo-some (human/ mouse)	Imprinted
Ligands				**Extracellular Antagonists**			
AMH (MIS)	464	19/10	No	BAMBI	30251	10/18	No
BMP10	20869	2/6	No	BMPER	24154	7/9	No
BMP15 (GDF9B)	1068	X/X	No	Chordin	1949	3/16	No
BMP2	1069	20/2	No	DAND5 (Coco)	26780	19/8	No
BMP3	1070	4/5	No	NBL1 (DAN)	7650	1/4	No
BMP3B (GDF10)	4215	10/14	No	Decorin	2705	12/10	Maternal for mouse, not human (verified, [21])
BMP4	1071	14/14	No	Follistatin	3971	5/13	No
BMP5	1072	6/9	No	Gremlin	2001	15/2	No
BMP6	1073	6/13	No	LTBP1	6714	2/17	No
BMP7 (OP1)	1074	20/2	No	Noggin	7866	17/11	No
BMP8A	21650	1/4	No	Sclerostin	13771	17/11	No
BMP8B (OP2)	1075	1/4	Predicted for humans	Twisted Gastrulation	12429	18/17	No

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GDF1	4214	19/8	No	**Receptors**			
GDF11 (BMP11)	4216	12/10	No	ACVR2B	174	3/9	No
GDF15	30142	19/8	No	ACVRL1 (ALK1)	175	12/15	No
GDF2 (BMP9)	4217	10/14	No	ACVR1 (ALK2)	171	2/2	No
GDF3	4218	12/6	No	ALK4 (ACVR1B)	172	12/15	No
GDF5 (BMP14)	4220	20/2	No	AMHR2	465	12/15	No
GDF6 (BMP13)	4221	8/4	No	BMPR2	1078	2/1	No
GDF9	4224	5/11	No	BMPRIA (ALK3)	1076	10/14	No
INHα	6065	2/1	No	BMPRIB (ALK6)	1077	4/3	No
INHβA (ACTA)	6066	7/13	No	TGFβR2	11773	3/9	No
INHβB (ACTB)	6067	2/1	No	TGFβR3	11774	1/5	No
INHβC (ACTC)	6068	12/10	No	**Intracellular Signal Transducers**			
INHβE (ACTE)	24029	12/10	No	SMAD1	6767	4/8	No
LEFTYA	3122	1/1	No	SMAD2	6768	18/18	No
LEFTYB	6552	1/1	No	SMAD3	6769	15/9	No
MSTN (GDF8)	4223	2/1	No	SMAD4	6770	18/18	No
NODAL	7865	10/10	No	SMAD5	6771	5/13	No
TGF-β1	11766	19/7	No	SMAD9(8)	6774	13/3	No
TGF-β2	11768	1/1	No	**Intracellular Inhibitors**			
TGF-β3	11769	14/12	No	SMAD6	6772	15/9	No
				SMAD7	6773	18/18	No
				SMURF1	16807	7/5	No
				SMURF2	16809	17/11	No

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