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Synexpression group analyses identify new functions of FSTL3, a TGFβ ligand inhibitor

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ABSTRACT

Follistatin-like 3 (FSTL3) is a secreted glycoprotein that forms inactive complexes with and acts as an endogenous inhibitor of TGFβ ligands such as activin, myostatin and GDF11. FSTL3 gene deleted mice (FSTL3 KO) are viable, fertile and show a constellation of metabolic abnormalities, including those involving glucose and lipid homoeostasis, suggesting a role for FSTL3 and TGFβ ligand signaling in these systems. To identify additional roles of FSTL3 and the ligands it inhibits we have used a synexpression analysis strategy. By mining microarray RNA expression data we have identified a group of 9 genes, the expression of which closely follow that of FSTL3 in both mouse and human tissues. After classifying the tissues studied according to physiological systems we found that within each system the expression of a majority, but not all, of the genes are strongly correlated with FSTL3 expression. Further, the best correlation of expression was seen in the cardiovascular system. Importantly, the promoter regions of a number of these synexpression genes have putative SMAD binding elements and in cultured embryonic fibroblasts the expression of a subset of these genes are induced in the absence of FSTL3 or in WT cells upon activin treatment. Taken together, we have identified a group of activin responsive genes the expression of which is closely related to and regulated by FSTL3. These findings link FSTL3 and TGFβ ligand signaling and a novel subset of the synexpression group of genes to organ/tissue-specific regulatory pathways.

1. Introduction

Tissue homeostasis is dependent upon the orchestration of multiple signaling pathways. The components of each signaling pathway might interact with other pathways, thereby creating a network of control mechanisms that is driven by multiple signals. TGFβ ligand signaling plays crucial roles in a large array of contexts including development, homeostasis and disease progression including, for example, cancer [1–6] and therefore regulates diverse outcomes such as organogenesis, glycaemic control, angiogenesis and cell division [7–10].

TGFβ ligand dependent signaling pathways appear predominantly linear involving ligand binding-mediated receptor kinase activation, subsequent phosphorylation and activation of SMAD transcription factors, which form active DNA binding complexes on TGFβ ligand responsive gene promoters and initiate a transcription response (reviewed in [11]). There is evidence that the ERK signaling pathway can modulate the TGFβ response [12,13] and new evidence suggests that TGFβ signaling might affect pathways such as p38 kinase signaling [14]. Together, therefore, these and other reports are evidence that TGFβ signaling mechanisms are open to cross-talk with other pathways, presumably to provide a concerted tissue homeostatis response. Hence, there exists the possibility that molecular components of the TGFβ ligand signaling pathways active in a given tissue might interact with others within the set of signaling networks relevant for that tissue.

TGFβ ligand signaling can be regulated by ligand-binding proteins that prevent ligands from interacting with their receptors. Follistatin like 3 (FSTL3) is such an endogenous glycoprotein that binds and inhibits TGFβ ligands such as activin, myostatin and GDF11 [15,16]. FSTL3 itself can be induced by activin, therefore completing a feedback inhibition loop that limits activin action in target cells [17]. While structurally and functionally similar to follistatin (FST), there are two major distinctions between FSTL3 and FST. FST is secreted and can be cell surface associated through binding to heparin sulphate while FSTL3 is secreted as well as nuclear [18,19]. Moreover, FSTL3 expression shows greater tissue specificity than FST [19]. Therefore although FSTL3 and FST bind and inhibit similar ligands, it is likely that the roles of FSTL3 in health and disease are distinct from those of FST [20].

The physiological functions of FSTL3 are not clearly understood but it is obvious that a component of these roles will involve activin-dependent signaling events. To identify the normal physiological functions of FSTL3 we generated FSTL3 gene deletion mice

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(FSTL3 KO) and found that, unlike FST KO mice, FSTL3 KO mice are viable and fertile [21,8]. Importantly, in our first study we found that the FSTL3 KO mice display an array of phenotypes that suggest crucial roles played by FSTL3, and therefore TGFβ ligand signaling in glucose and lipid homeostasis [8]. These novel roles of FSTL3 were unexpected and therefore suggest the possibility that FSTL3 action might be crucial to other homeostatic and organ systems.

To identify additional physiological roles of FSTL3, and therefore infer roles of activin and related ligands that FSTL3 inhibits, in tissue homeostasis, we have adopted an *in silico* data mining approach with the following logic. First, it is likely that the physiological roles of FSTL3 will be dictated by the functional processes most relevant in the tissue/organ that FSTL3 is expressed in. Secondly, genes that follow an expression pattern closely matched with that of FSTL3 are likely to form a synexpression group of genes that might function individually alongside or in concert to produce one or more homeostatic outcomes reviewed in [22]. Finally, while the synexpression genes might belong to different homeostatic pathways, their expression will be affected by FSTL3 expression if these pathways and FSTL3 dependent pathways crosstalk and the hierarchical position of the synexpression genes in these pathways is subsequent to the point in the pathway where the FSTL3-dependent pathway can crosstalk (Supplementary Fig. 1). This approach allows us to significantly extend and refine our understanding of the signaling roles not only of FSTL3 and activin but also the regulation of the synexpression group of genes. It also highlights any possible opportunities for crosstalk between the FSTL3-activin-SMAD pathway and other homeostatic signaling pathways in an organ/tissue-specific manner.

2. Materials and methods

2.1. Data mining

Searches were performed on EBI ArrayExpress, a repository of microarray RNA expression data (http://www.ebi.ac.uk/arrayexpress/). Using FSTL3 as the search "keyword" 45 experimental data sets were found that include fstl3 expression data. Of these results, E-AFMX-5 dataset (Transcription profiling of human cell lines and tissues) from GNF/Novartis was used for further analysis. From this data set, the identities of 10 genes were queried, including FSTL3, which showed closest correlation in RNA expression patterns. The results were further refined by performing the same search on data from the experiment E-MTAB-24 (transcription profiling of human and mouse organism parts where organism parts are exactly matched between the two species). Expression data, normalized to internal controls, from this search were extracted as a worksheet and converted into Microsoft Excel format for further analyses.

2.2. Cell culture

Mouse embryonic fibroblasts were isolated from 14.5 day old WT and FSTL3 KO mouse embryos. Upon isolation, embryos were washed in PBS and minced using a fine scalpel before being trypsinized. Resuspended cells were passed through a 70 μm cell strainer (Costar) before being plated in fresh DMEM (Sigma, UK), 10% heat-inactivated fetal bovine serum (Gibco, UK), L-glutamine (Gibco, UK), and antibiotics (100 U/ml penicillin and 10 μg/ml streptomycin; Gibco, UK). Cells were maintained in DMEM until they reached 80% confluence. WT cells were either treated with 25 ng/ml activin (R&D) for a further 24 h or left untreated.

2.3. RNA Isolation and RT-PCR

Total RNA was extracted from cultured cells with TRIzol (Invitrogen, Carlsbad, CA) following manufacturer's directions. 1 μg of total RNA was reverse transcribed (Applied Biosystems High Capacity cDNA kit) and cDNA were used for conventional PCR using, specific primer sets and Promega GoTaq kit on a BioRad DNA Engine Tetrad 2 PCR machine. PCR products were size separated on 1% Agarose TAE/ethidium bromide gels and imaged using a BioRad gel-documentation system. The specific fluorescent bands were quantified by densitometry using NIH ImageJ. Specific message concentrations were normalized to mouse ribosomal protein L19 (RPL19).

3. Results

3.1. Identification of FSTL3 synexpression group

Searching through microarray experimental datasets we first obtained the FSTL3 mRNA expression profile for a large collection

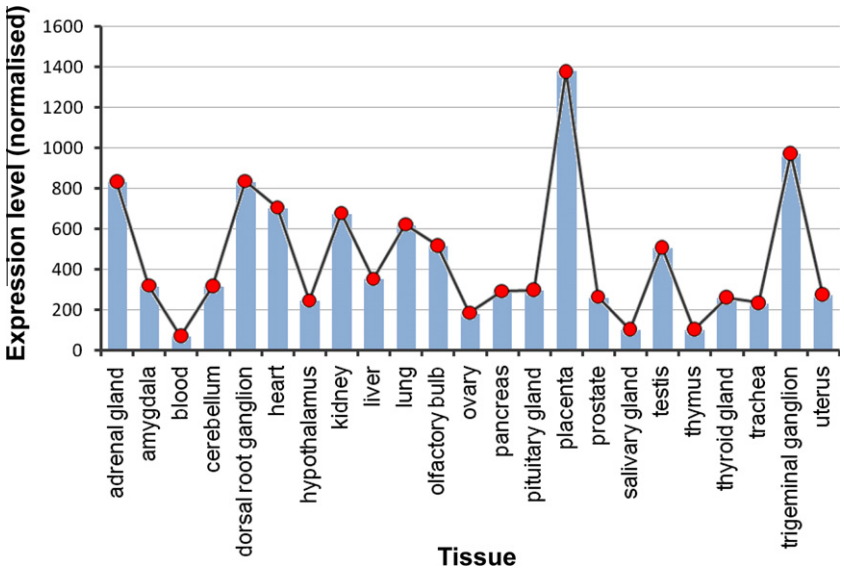

Fig. 1. FSTL3 expression is spatially regulated. Bar graph and interpolated line graph showing expression levels of FSTL3 in tissues indicated, as mined from microarray data. FSTL3 expression shows strong tissue-specific regulation.

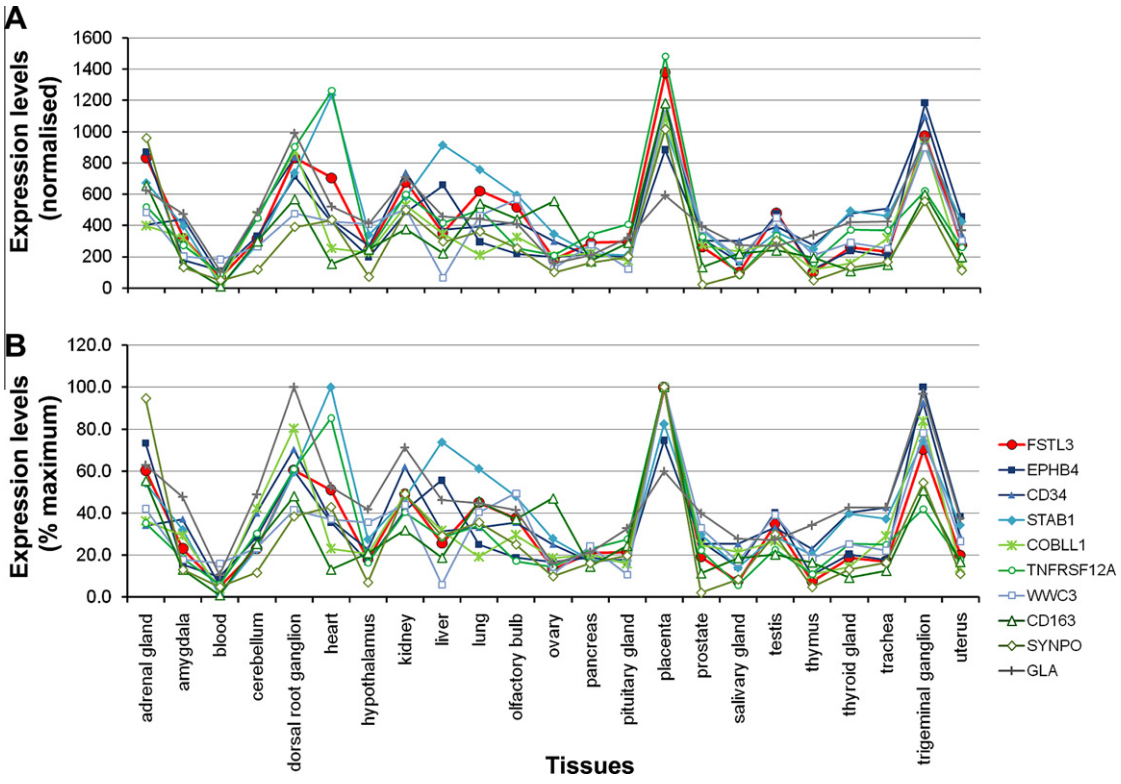

Fig. 2. FSTL3 synexpression group. The expression profiles of 9 genes presented as line graphs, that match FSTL3 expression most closely in indicated tissues. Data are shown as either normalized to internal control as obtained from the data repository (A) and normalized data calculated as a percent of the maximal expression in a single tissue for each transcript (B).

of tissues (23, Fig. 1). It is evident that FSTL3 shows remarkable tissue specificity with the highest expression in placenta and lowest in blood cells (Fig. 1). We then identified a synexpression group of 10 genes including FSTL3. While it is possible to increase the group size to include more gene transcripts in the analysis, it is likely that the transcripts most closely aligned with FSTL3 expression will be most informative regarding the function of FSTL3 and TGFβ ligand signaling. Also, among the possible relationships between two transcripts, we chose to focus on genes with expression profiles that correlate positively with FSTL3 expression. The experimental data obtained from mining the database is normalized to internal housekeeping gene controls. The expression patterns of this group of 10 genes (normalized to controls) are shown in Fig. 2A. A majority of these genes show highest expression in the placenta, with the exception of *EphB4*, *Stab1* and *Gla* that show highest expression in trigeminal ganglion, heart and dorsal root ganglion, respectively. Since the normalized expression level varies between genes, to get a better representation of the relevance of each gene for the function of tissues tested, the normalized expression is expressed as percent maximal and represented in Fig. 2B.

3.2. Tissue-specificity of FSTL3 synexpression group

It is difficult to estimate the minimal gene expression level necessary for significant contribution to the specialized function of tissue/organ. There are however, tissue specific functions for many genes: the more tissue-specific or selective the gene function is, the fewer the number of tissues in which it is expressed at high levels. For ease of analysis and to focus our studies we chose an arbitrary cut-off of 15% maximal expression for each transcript with the assumption that an expression lower than 15% maximal for a transcript in a given tissue indicates that the importance of the respective gene in terms of organ-specific function is low that tissue. With this criteria we find that SYNPO and CD163 function is relevant in about only about 50% and 60% of the tissues studied, respectively (Fig. 3A) and that this synexpression group, in total, is most relevant for adrenal gland, dorsal root ganglion, kidney, liver, lung, olfactory bulb, placenta, testis and trigeminal ganglion (Supplementary Fig. 2). This analysis however excludes the possibility that genes within the synexpression group might play crucial roles in tissues where they are expressed independent of the status of FSTL3 expression. There appears to be a good correlation of expression of these synexpression transcripts with neuronal, cardiovascular and reproductive/endocrine tissues and their functional importance based on expression profiling perhaps bears least relevance to circulating blood cells.

To further investigate this we measured the correlation of the expression between each of the transcripts and that of FSTL3 across 10 tissues that have at least 25% of maximal expression of FSTL3. In doing so, the analysis was focused to 10 tissues. These tissues were classified into three physiological systems: neuronal tissues (dorsal root ganglion, trigeminal ganglion and olfactory bulb), cardiovascular tissues (placenta, heart, kidney and lung) and endocrine tissues (adrenal gland, testis and liver). The correlation of expression of each transcript with FSTL3 in these systems was then analyzed. These findings are summarized in Supplementary Table 1 and shown graphically in Fig. 3B. While most of the transcripts appear well correlated with FSTL3 expression across all the systems there are a few crucial differences. STAB1 was least correlated with FSTL3 expression in the cardiovascular tissues. GLA showed a variable range of correlation with FSTL3 expression with the highest correlation in neuronal tissues and TNFRSF12A was best correlated in endocrine tissues.

Taken together therefore, FSTL3 shows significant expression in cardiovascular, neuronal and endocrine tissues and the group of synexpression genes examined shows highest correlation with

FSTL3 expression in neuronal tissues. Importantly, barring the three transcripts mentioned above, expression of most transcripts within the group of genes identified are correlated well with FSTL3 expression across tissues.

3.3. SMAD binding elements in synexpression gene promoters

Given that FSTL3 is an inhibitor of activin and FSTL3 expression itself is activin responsive, it is possible that a subset of the synexpression genes might be activin responsive. To assess this possibility we examined the promoter regions of these genes for the presence of canonical SMAD binding elements (SBE). As shown in Supplementary Table 2, *EphB4*, *Cd34*, *Cobll1*, *Stab1* and *Gla* has at least one SBE in the proximal region (within 1 kb) of the promoter. There are no SBEs within 3 kb upstream of the start codon of *Tnfrs12a*, *Wwc3*, *Cd163* and *Synpo*. It is likely therefore that five of the FSTL3 synexpression group genes are activin responsive.

To directly test whether these five genes (*EphB4*, *Cd34*, *Cobll1*, *Stab1* and *Gla*) are indeed activin responsive, mouse embryonic fibroblasts (MEF) isolated from mice carrying the Floxed FSTL3 allele, with intact FSTL3 coding region, were either treated with 25 ng/ml activin for 24 h or left untreated. Total RNA isolated from these cells was assayed for the expression of the above genes using RT-PCR. To test how the expression of FSTL3 affected these genes we also assayed the expression in MEFs isolated from FSTL3 KO mice. As shown in Fig. 4, the expression of CD34, EPHB4 and STAB1 were induced by activin treatment, COBLL1 expression was modestly upregulated whereas GLA expression was unaltered, much like the expression of TNFRSF12a, a gene without putative SBEs in its promoter. Importantly, the expression of almost all of the genes tested in FSTL3 KO MEFs mirrored those in activin treated Floxed MEFs, with the exception of COBLL1, which is reduced in comparison to untreated Floxed MEFs.

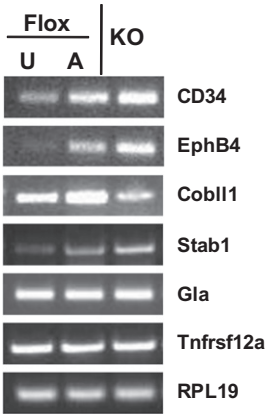

Fig. 4. Activin induces a subset of FSTL3 synexpression group genes. Photographs of ethidium bromide stained agarose gels showing RT-PCR amplification of indicated transcripts in RNA isolated from untreated (U) or activin (A) treated FSTL3 floxed (Flox) or FSTL3 KO (KO) mouse embryonic fibroblasts. RPL19 was used as internal control.

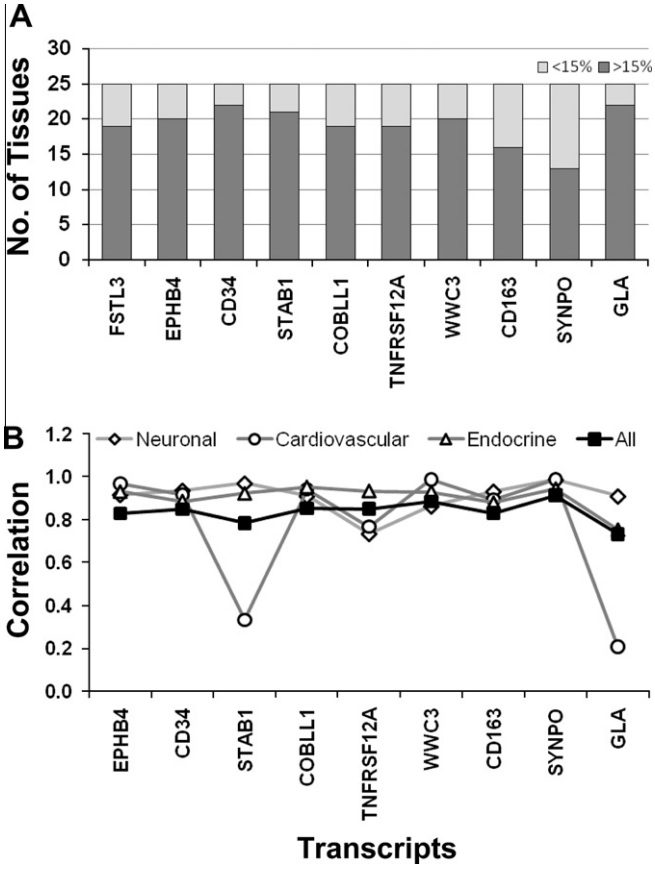

Fig. 3. Tissue and system specific correlation of FSTL3 synexpression group genes. (A) Bar graph showing the number of tissues in which each of the transcripts are expressed, considering % of maximal expression as greater or less than 15%, in each tissue. (B) The correlation of the synexpression genes with FSTL3 expression in neuronal, cardiovascular, endocrine or all tissues examined.

4. Discussion

Activin, a member of the TGFβ family of ligands plays crucial roles in numerous cellular functions and developmental contexts. While there is an extensive literature on activin action it is difficult to ascertain the specific mechanisms by which activin signaling affects cellular, tissue and organismal physiology. Moreover, it is difficult to predict the target tissues where activin action might be important. Activin subunit gene specific mouse deletion models have demonstrated that activin expression is essential for life and important in craniofacial development [23], however, the extent of effects, or phenotypes associated with of a lack of activin action across a large collection of tissues representing multiple organ systems have not yet been established. The cumbersome nature of using multiple gene deletion models to perform tissue specific phenotype analysis is perhaps the major reason why progress has been slow to this end. The situation with respect to other TGFβ ligands, most of which appear to be pleiotropic, is the same.

Moreover, the TGFβ signaling pathway appears to be largely linear with a high degree of promiscuity in the signaling output: either SMAD1/5/8 or SMAD2/3 mediated gene transcription in response to a large number of ligands [11]. One of the mechanisms of regulating the TGFβ ligand response is by regulating the temporal and tissue specific expression of the ligands, ligand receptors and also ligand inhibitors. Among the ligand inhibitors follistatin (FST) and FSTL3 are very closely related structurally and hence functionally they can both inhibit a common set of ligands. Interestingly, however, while they have very similar primary and 3-dimensional structures, FST is broadly expressed whereas FSTL3 shows significant tissue selectiveness [19]. This therefore suggests that not only are these two ligand traps non-redundant, but also that FSTL3 might play more selective or fine tuning roles, as far as activin signaling is concerned, in tissues expressing FSTL3.

Activin can act in both a paracrine and endocrine manner and induces FSTL3 in target tissues [17]. By following FSTL3 expression, therefore, it is possible to identify a subset of activin target

tissues. Furthermore, classification of these tissues into tissue systems enables us to ascribe roles, known or novel, to activin and FSTL3 in physiological systems. Genes that show expression profiles similar to FSTL3, are thus likely to be either activin targets or relevant to a particular physiological system where activin and FSTL3 are also important. Following this logic we find FSTL3 expression is closely linked to cardiovascular, neuronal and endocrine related organs. While TGFβ ligand signaling *per se* has been shown to be important in the cardiovascular system [24], here we identify activin, specifically, to be relevant to this system. Most interestingly our findings suggest novel roles of activin and FSTL3 in neuronal systems. This is a crucial finding that will lead to future investigations of the role of activin and FSTL3 in neuronal development and function.

Here we also report the identity of 9 genes, the expression patterns of which are closely aligned with that of FSTL3, that are therefore putatively associated with the activin-FSTL3 signaling pathway. The method used to identify these potentially key mechanistic players has also revealed previously unknown functional roles for several gene products. For example, the result that synaptopodin (SYNPO) is strongly expressed in the placenta, suggests a role for this molecule in placental biology. SYNPO has hitherto been linked to kidney and neuronal functions [25], tissues where it is expressed highly, but at relatively lower levels than the placenta. Hence, by association with FSTL3 expression we have identified possible target tissues, some corresponding to novel functional roles, for the 9 synexpression genes.

In relation to the cardiovascular system FSTL3 has been shown to be important in the heart [26] as well as very highly expressed in preeclamptic placenta [27]. It is not clear whether increased FSTL3 expression is the cause or remedial response in the preeclamptic placenta. Recent evidence shows that EphB4 is significantly downregulated in preeclamptic placenta in a microRNA dependent mechanism, therefore suggesting an important role for EphB4 in placental function [28]. This supports our analysis that suggests the relevance of EphB4 expression in cardiovascular tissues.

Since FSTL3 is induced by activin in target cells it is possible that a subset of the FSTL3 synexpression genes is also activin responsive. Indeed we found putative SBEs in 5 of the 9 synexpression genes. While the mere presence of SBEs does not necessitate activin-responsive expression, this analysis enabled a narrowing down of the candidate genes that might be activin responsive. Our subsequent *in vitro* experiments using MEFs treated with activin showed that of these 5 genes, CD34, EphB4, Cobll1 and Stab1 are indeed activin inducible therefore while Gla is not. Interestingly in MEFs, although Cobll1 is activin responsive, FSTL3 KO MEFs do not show increased Cobll1 expression perhaps due to the innate properties of the FSTL3 KO fibroblast cell. Stab1 and Gla are least correlated with FSTL3 expression in the cardiovascular system. So although Stab1 is activin-responsive it is possible that neither Stab1 nor Gla are essential components of or associated with the activin-FSTL3 signaling pathways in cardiovascular tissue.

Taken together therefore, using a method of a synexpression group analyses we have identified novel directions to study activin dependent physiology, novel target tissues of gene action and a set of novel activin responsive genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.098.

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