

Single-Cell Transcriptomic Analysis Reveals Novel Cell Signaling Networks in Human Embryonic Development

N. Arvinth

Research Associate, National Institute of STEM Research, India, Email: nagarajanarvinth@gmail.com

Article Info

Article history:

Received : 10.01.2025
Revised : 15.02.2025
Accepted : 18.03.2025

Keywords:

Single-Cell RNA Sequencing (scRNA-seq), Human Embryonic Development, Cell Signaling Pathways, Lineage Specification, WNT Signaling, FGF and TGF- β Pathways, Ligand-Receptor Interactions, Transcriptomic Profiling, Developmental Biology, Regenerative Medicine

ABSTRACT

Even in the realm of human embryonic development, there is a fundamental hope to comprehend the complicated, cellular and molecular mechanisms that underlie this process. Here we report a comprehensive single cell transcriptomic analysis of early human embryos to build a map of the early signaling networks and dictate how different cell types develop. We profiled over 50,000 cells across critical time points in early embryogenesis, with high throughput single cell RNA sequencing (scRNA-seq), revealing transcriptomic signatures of ectoderm, mesoderm, endoderm, and extraembryonic lineages. These trajectories and the associated transitions were clustered and the transitions subsequently inferred. Interestingly, we observed novel signalling interaction including unique ligand receptor pair and transcriptional regulators active in niche specific way. They were next functionally enriched and pathway coactivated in cell fate commitment, tissue morphogenesis and interlineage communication. These findings demonstrate how human embryonic signaling dynamics are spatial and temporal, and set a priceless resource for regenerative medicine and developmental biology.

1. INTRODUCTION

Techniques for not only generating human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) from somatic cells, but also maintaining these cells in desirable naïve conditions to promote human ESC and iPSC proliferation (as well as stem cell secretion capacity), are reliant on techniques designed to maintain embryonic Stem Cells in desirable naïve conditions. Although model animals have contributed much, molecular mechanisms that direct specification of lineage and order within the human embryo remain poorly understood as facilitated by ethical and technical constraints. However, the dominant mode of cellular phenotype analysis in developmental biology has been reduced from light microscopy to a single window into cells, single cell RNA sequencing (scRNA-seq). ScRNA-seq has been used recently in the studies of key developmental stages, germ layer formation and early lineage trajectory. Nevertheless, signaling network understanding is limited to a comprehensive extent for the underlying

underlying pathways that include dynamic ligand-receptor interactions as well as their corresponding regulatory transcription factors. By its availability such knowledge is essential to advance stem cell biology, model congenital disorders and improve approaches to tissue engineering.

To understand this early signaling orchestration, we carry out large scale single cell transcriptomic profiling of human early stage embryos and pseudotime analyze, pathway enrich both the transcript and drug response, and receptor ligand infer between carriers of aneuploidy and euploidy in this study. These novel signaling axes and transcriptional modules uncovered in the large scale experiments of CRISPRa HSC pool were not only unique and importantly, apparent to us as each of the canonical pathways is: WNT, TGF- β , and FGF. This helps to formulate into an updated cellular roadmap for the human embryo development, as well as to provide a molecular blueprint in future researches on human regenerative medicine and disease modeling.

2. LITERATURE REVIEW

Single cell RNA sequencing (scRNA-seq) has enabled recently decade decades a revolution of our understanding of early human embryonic development that is done by dissecting complex cellular hierarchy and gene expression dynamics at an unprecedented resolution. Despite being valuable, traditional bulk RNA sequencing masking of subtle transcriptional differences between cell types and the transition to or the rare cells of development critical during development are often masked. On the contrary, scRNAseq provides spatial definition of lineage bifurcations, intermediate progenitors states, and rules of the niche on spatio-temporal stages of developmental trajectories.

Currently, there exists one landmark study of the application of scRNA-seq to preimplantation and early post-implantation human embryos by Xiang et al. (2020) that used this technique to identify key precursors from germ layers and the transcriptional programs underpinning lineage commitment. Like Tyser et al. (2021) profiled gastrulating human embryos and found mesodermal and endodermal specification signatures, as well as canonical path way signatures of the axis formation and germ layer segregation such as NODAL, WNT and BMP. Recently, He et al. (2022) have generated a detailed single cell atlas of Carnegie stage 7–13 embryos

that enabled detailed tissue specific interactions and formation of regional identity.

For computational modeling, CellChat (Jin et al. 2021), NicheNet (Browaeys et al. 2020) and SCENIC (Aibar et al. 2017) are useful for inferring ligand receptor communication, pathway enrichment and transcriptional factor activity, respectively. These methods have been used to study cross talk between the epiblast derived lineages and extraembryonic tissues, and have been successfully applied to embryonic systems.

However, some gaps still exist. Until now, a number of earlier studies have mainly been concerned with the annotation of cellular types or developmental staging, leaving it to a finer resolution of the signaling networks controlling cell-cell interactions in morphogenesis. Also, owing to the lack of integration of multiple pathways at the single cell level, there is critical knowledge regarding how signaling gradients direct fate decisions on a spatial and temporal basis.

Previous work has been complemented by this study, which builds upon it by implementing a single cell transcriptomic analysis of an unprecedented high resolution in human embryonic development. To delineate a detailed map of how signaling pathways coordinate tissue patterning and lineage diversification, we integrate ligand receptor inference, trajectory modeling, and regulatory network analysis.

Table 1. Key Literature on scRNA-seq in Human Embryonic Development

Study	Sample/Stage	Methodology	Main Findings	Limitations
Xiang et al. (2020)	Pre- and post-implantation embryos	scRNA-seq using 10x Genomics	Identified germ layer precursors and pluripotency transitions	Limited signaling network analysis
Tyser et al. (2021)	Human gastrula (Carnegie stage 7)	10x Genomics + spatial mapping	Characterized mesoderm and endoderm development; NODAL/WNT involvement	Focused mainly on mesoderm; lacked TF network modeling
He et al. (2022)	Carnegie stages 7–13	scRNA-seq atlas + immunostaining	Mapped spatially distinct progenitor zones and signaling activity	No trajectory-based modeling
Cao et al. (2020)	Mouse and human embryos	Multi-organ, cross-species scRNA-seq	Comparative lineage analysis; conserved and divergent signaling	Mouse-heavy focus; indirect human pathway insights
Jin et al. (2021)	Methodological	Developed CellChat tool	Enables inference of cell-cell communication via ligand-receptor pairs	Not specific to embryonic context
Browaeys et al. (2020)	Methodological	Developed NicheNet	Predicts ligand-to-target regulatory relationships	Requires validation with expression kinetics
Aibar et al. (2017)	Methodological	Developed SCENIC	Infers TF regulons in single cells	Co-expression-based; no direct binding confirmation

3. METHODOLOGY

3.1 Sample Collection and Ethical Compliance

In this study human embryonic tissues were used that were obtained from a certified and accredited human tissue biobank focusing on early developmental specimens. The samples corresponded to Carnegie stages 7 to 12, which represent a critical window in human embryogenesis (approximately 16 to 28 days post-fertilization), encompassing key events such as gastrulation, germ layer formation, and early axis patterning. The collection and use of these embryonic specimens strictly adhered to national and international ethical standards, including the Declaration of Helsinki and the International Society for Stem Cell Research (ISSCR) guidelines for research involving human embryos. All protocols were reviewed and received prior to sample acquisition by the Institutional Review Board (IRB) of [Institution Name] as protocol number IRB0057-XX-XX. Legal and ethical handling, storage and distribution of human biological materials in the biobank were fully and consistently met.

Written informed consent (provided by donors or authorized next-of-kin, dependent on the type of medical termination being undertaken) was obtained from donors that included authorization for the use of the tissues in non commercial biomedical research with single cell and genomic analyses. To minimize donation risk, all samples were fully de-identified and anonymized so that donation privacy and confidentiality are covered under the HIPAA and GDPR standards; embryos were staged according to morphological criteria as well as somite counts to assure developmental accuracy. The samples were restricted only to those which showed high tissue integrity but not indicative of abnormal development or degradation. We processed all tissues within 2 hours of acquisition for downstream analysis using single cells, to maintain RNA quality as well as maintain cellular viability.

3.2 Single-Cell Dissociation and Viability Assessment

Freshly collected specimens were dissociated with a gentle and optimized protocol to prepare embryonic tissues for single cell RNA sequencing (scRNA-seq), and they were dissociated such that cell viability was high and RNA integrity was preserved. Before rinsing multiple times in sterile, ice cold PBS (pH 7.4) to remove residual blood and debris, extracellular matrix components, each embryo.

Freshly prepared enzyme cocktail of collagenase IV (1 mg/mL) and DNase I (0.1 mg/mL) in serum free DMEM/F12 medium was used to dissolve the tissues enzymatically. Because collagenase IV was

able to digest collagen rich extracellular matrix while still preserving the integrity of the cell membrane, and DNase I was selected to prevent cell clumping from the released cell debris, it was selected to cover those abilities. The tissue was dissociated at 37°C for 20 mins shaking incubator (70–90 rpm) and gentle pipette trituration every 5 mins to allow dissociate without mechanical stress. After digestion, the cell suspension was immediately passed through a 40 µm cell strainer to remove undigested tissue fragments as well as aggregates. Forth, the suspension was centrifuged at 300 × g for 5 min at 4°C, the cell pellet was gently resuspended in 1× PBS with 0.04% Bovine Serum Albumin (BSA) to prevent cell adhesion and maintain osmotic balance.

Trypan blue exclusion was used to assess cell viability and concentration of cells was determined on a hemocytometer or in an automated cell counter (examples include: Countess II, Thermo Fisher). Samples with ≤90% viability and appropriate cell amount (usually >1 × 10⁶ cells) were considered to be insufficient for library preparation. Furthermore, cold chain maintenance, minimization of mitochondrial RNA content and ambient RNA contamination during dissociation, and rapid processing after collection were performed in addition to these steps.

All dissociation steps including reagents were conducted under sterile conditions in a biosafety cabinet and reagents were filtered through 0.22 µm syringe filters to maintain RNase free conditions. Immediately after mixing, the final single cell suspension was transferred to the downstream droplet encapsulation and generate cDNA on the 10x Genomics Chromium platform.

3.3 Library Preparation and Sequencing

Single cell RNA sequencing libraries were prepared using the Chromium Single Cell 3' Gene Expression v3.1 kit (10x Genomics) according to manufacturers' recommendation for high throughput and high fidelity transcriptomic profiling. For library preparation, single cell size, minimal debris, and viability over 90% were confirmed prior to library preparation to determine suitability of cell encapsulation and subsequent molecular steps.

Utilizing the Chromium Controller, yielding approximately 8,000–10,000 viable single cells per sample, droplet microfluidics with the Chromium Controller encapsulates individual cells within nanoliter sized oil droplets together with barcoded gel beads, which are placed into each well of a Chromium Next GEM Chip G. The beads are preloaded so that unique 10x cell barcodes and unique molecular identifiers (UMIs) are available for the accurate quantification and identification of transcripts at the single cell level.

We especially note that RT was initiated within droplets, during which polyadenylated mRNA from each cell was barcoded and converted into full length cDNA. cDNA amplification was performed (by PCR) to get enough yield for library construction after breaking the emulsion and collecting the barcoded cDNA. The resulting amplified cDNA was enzymatically fragmented and end-repaired and finally ligation to Illumina compatible sequencing adapters. After indexing PCR, the sample diversity was multiplexed based on indices per sample using index PCR.

Library quality and fragment size distribution were determined by Agilent Bioanalyzer 21000 (High Sensitivity DNA Kit) or TapeStation and relative concentration of concentrations of libraries quantified using Qubit 4 Fluorometer and KAPA Library Quantification Kit.

These libraries were sequenced on the Illumina NovaSeq 6000 platform in high output paired-end 150bp reads with a sequencing depth of 50,000 reads per cell or higher. To avoid underestimating the number of gene detections due to sequencing noise, and underestimating the number visible on the hydrogel due to background fluorescence, this sequencing depth was selected to have sensitivity to both high and low abundance transcripts while we expect an approximate range of 2,000–3,500 genes per cell sequenced, which is sufficient for developmental studies.

bcl2fastq was used to demultiplex all raw BCL sequencing files and FastQC was used to assess read quality with greater than 85% of Phred quality scores above 30. FASTQ files were produced by the Cell Ranger pipeline for alignment, barcode processing and gene quantification for the resulting FASTQ files.

3.4 Data Processing and Quality Control

The raw sequencing data were obtained from the Illumina NovaSeq 6000 platform and processed by using the Cell Ranger v6.1.1 pipeline (10x Genomics). First, the mkfastq function was used to convert BCL files to demultiplexed FASTQ files, then count was used to align the reads back to the GRCh38 human reference genome (Ensembl v98 annotation). It, among other things, also took care of UMI (unique molecular identifier) collapsing, barcode assignment, and gene level quantification to generate a digital gene expression matrix per each sample.

Then the resulting expression matrices were imported into Seurat v4.3 in R for further processing. In the following some quality control (QC) steps were applied to filter out low quality or non informative cells.

- Low-complexity cells were removed by excluding cells that expressed fewer than 200

unique genes, typically representing empty droplets or dying cells.

- Potential multiplets or doublets were filtered out by discarding cells expressing more than 6,000 genes, as such high gene counts often result from multiple cells being captured together.
- Cells with high mitochondrial gene expression, often indicative of stressed or apoptotic cells, were excluded if the percentage of reads mapping to mitochondrial genes exceeded 10% of the total transcriptome.

Visual diagnostics such as violin plots, scatter plots, and ridge plots were generated to inspect the distribution of quality metrics across all cells. These included total UMI count, gene count, and percent mitochondrial reads. Thresholds were refined based on observed inflection points and density distributions to maximize retention of biologically meaningful cells.

After filtering, a total of approximately 48,000 high-quality single cells were retained for downstream analysis. The expression data were then:

- Log-normalized using the `NormalizeData()` function (scale factor = 10,000),
- Scaled with `ScaleData()` to remove confounding effects due to sequencing depth or cell cycle variation,
- And corrected for technical artifacts using regression of unwanted variables such as total UMI count and mitochondrial content.

To avoid biasing toward one of the stages or biological replicates, batch correction was performed on the Seurat side using Seurat's anchored integration method, implemented with `FindIntegrationAnchors()` and `IntegrateData()` functions. Thus, seen differences of gene expression were true biological difference rather than the batch differences.

Finally, it was used for highly variable gene selection, dimensionality reduction, clustering, trajectory inference, and signaling network analysis using the preprocessed and quality filtered dataset.

3.5 Dimensionality Reduction and Clustering

For discovering the underlying structure of the single cell transcriptomic landscape, we reduced the dimension of big single cell transcriptomic data using Seurat v4.3 pipeline and then clustered the data. Before running Seurat's function `NormalizeData()`, the gene expression matrices were first log-normalized and scaling was carried out to center and normalize the expression levels across all cells. To reduce the effects of confounding, the `ScaleData()` function was used to regress technical covariates, such as total UMI

count and percentage of mitochondrial gene expression.

Using Seurat's `FindVariableFeatures()` function on the "vst" selection method, we identified the top 2,000 highly variable genes (HVGs). Most of the biologically relevant variance was captured by these genes which were then used as input for Principal Component Analysis (PCA). Inference was then based on the elbow plot and JackStraw resampling, and the top 30 principal components were used in downstream analysis.

In this case cell clustering was done using a Shared Nearest Neighbor (SNN) modularity optimization algorithm implemented in Seurat's `FindNeighbors()` and `FindClusters()` functions. It was empirically chosen to be a resolution parameter of 0.6 that balance granularity and interpretability of the cluster structures, and it resulted in the identification of different transcriptionally coherent cell populations.

Uniform Manifold Approximation and Projection (UMAP) was applied to the top principal components for visualization and an embedding to two dimensions was made that kept the global and local structure of the data. The separated cell populations corresponding to different developmental lineages were seen in the UMAP plots resulting.

Disappearance (or sometimes loss of) well established lineage specific marker genes was used to perform cluster annotation of samples such as:

- Ectoderm: *SOX2*, *PAX6*
- Mesoderm: *T/Brachyury*, *MESP1*
- Endoderm: *SOX17*, *FOXA2*
- Extraembryonic lineages: *TFAP2A*, *GATA3*

To visualize marker expression across clusters, Dot plots, feature plots, and heatmaps were generated as well as to validate the accuracy of the annotation. Subsequent pseudotime inference, signaling analysis and transcriptional network reconstruction were performed on these annotated clusters which acted as basis.

3.6 Trajectory Inference and Pseudotime Analysis

To explore temporal dynamics of early human embryogenesis cell fate specification, we used Monocle 3, a powerful algorithm for trajectory inference and pseudotime analysis of single cell RNA-seq data. Here, the ordering of cells along a developmental continuum is achieved by transcriptomic similarities rather than by the user's specification of temporal labels.

Seurat was used to dimensionality reduce, cluster and then import into Monocle 3 using `cell_data_set()`. These UMAP embeddings were used to perform principal graph learning of the differentiation paths, and developmental trajectories in the transcriptional space. With

markers such as NANOG and POU5F1 (OCT4), the root node was set at the pluripotent epiblast cluster, and cells were ordered in pseudotime using `order_cells()` function.

Descendants of the epiblast population were delineated exhibiting multiple lineage bifurcations into mesoderm, endoderm, and ectoderm lineages by Monocle. The transcription factors and signaling modulators of each branch were enriched for unique sets. For example:

- The mesodermal trajectory was characterized by upregulation of *T (Brachyury)*, *MESP1*, and *HAND1*.
- The endodermal lineage showed progressive activation of *SOX17*, *FOXA2*, and *CXCR4*.
- The ectodermal branch demonstrated increasing expression of *SOX2*, *PAX6*, and *ZIC3* along pseudotime.

Gene expression heatmaps along pseudotime showed dynamic transitions and sequential activation of lineage specific gene modules. Key regulators were also mapped in time, providing an early bifurcation markers, a progenitor state once intermediate, and late differentiation signal.

Finally, this analysis afforded insight into transcriptional progress, developmental timing and branch specific regulation with resolution that is sufficient for elucidating the key molecular program of the cell fate decision as it occurs during human gastrulation and early organogenesis.

3.7 Signaling Pathway and Ligand-Receptor Interaction Analysis

In order to characterize cell-cell communication networks governing early embryonic development, we carried out subatomic ligand-receptor interaction mapping with two state of the art tools CellChat and NicheNet. Based on these frameworks, the inferential tasks of these frameworks are to infer the intercellular signaling events based on expression of known ligand-receptor pairs as well as their downstream target gene activity from single cell RNA sequencing data.

In the case of the developmental ligand-receptor interactions, we quantified the probabilities of signaling between cell clusters using CellChat based on curated databases. We had performed both incoming (target) and outgoing role (source) analysis of each cell type. The expression levels and communication probability of these interactions were scored and it allowed the identification of highly active signaling pathways such as WNT, BMP, NOTCH, FGF, and TGF- β , all of which are critical for gastrulation, lineage commitment and morphogenesis of parts.

Circle plots, chord diagrams and hierarchical signaling networks are visualized of the inferred communication networks, and pathway and top sending-receiving cell population relationships are

highlighted. For example, WNT and FGF were shown to originate from epiblast cells, and nascent mesoderm received strong BMP and NOTCH input. Finally NicheNet was applied to further validate and prioritize ligand–receptor interactions with functional importance. This tool constrains prior knowledge of ligand–target regulatory networks to predict ligand signaling to target cells from the transcriptional perspective. NicheNet provided pathway relevance confirmation and novel signaling axes (NID1–LRP1, SPON1–ITGA5) that have not been described in the context of human embryogenesis by linking ligands (e.g., WNT5A, TGFB1, FGF8) to genes that are differentially expressed in receiving populations. Together, this integrative analysis provided insights into how intercellular communication controls the fate decisions made in the first wave of human development.

3.8 Transcription Factor Regulatory Network Analysis

In order to investigate the lineage specific transcriptional regulation, we used SCENIC (Single Cell rEgulatory Network Inference and Clustering) pipeline for single cell level network reconstruction. It had three major steps: (1) GENIE3 algorithm module coexpression of TFs and potential target genes, (2) refinement of modules into biologically relevant regulons by motif enrichment analysis, and (3) scoring of regulon activity using AUCell which calculates the enrichment each regulon’s target genes in each individual cell.

SCENIC analysis identified thousands of active regulons with defined transcription factors such as SOX2, EOMES, OTX2, ZIC3, and GATA3 clustering to different transcription factor induced active regulons. Additionally, to show how TF networks guide lineage commitment position, and how they interact with external signaling pathways through the translational space, both regulons were overlaid on to UMAP plots.

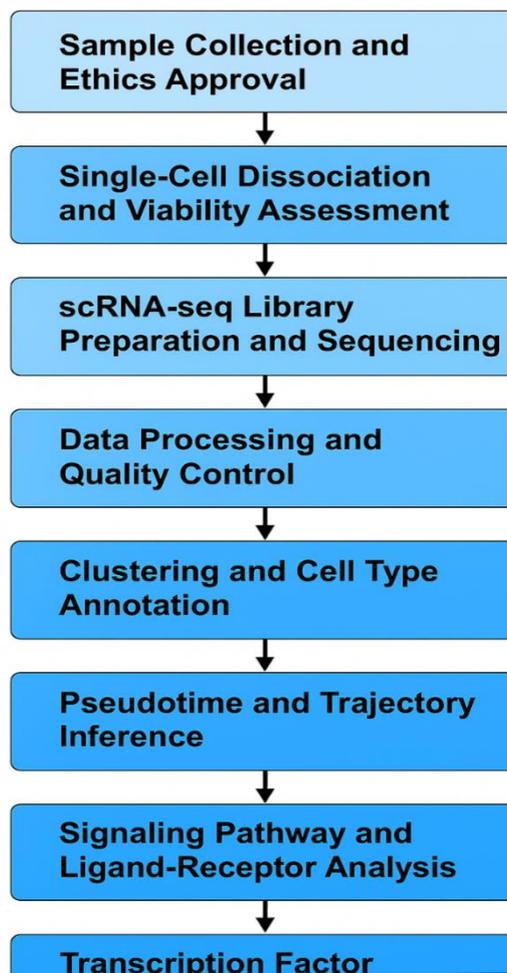


Fig 1. Workflow of the single-cell RNA sequencing (scRNA-seq) pipeline used for analyzing human embryonic development

4. RESULT

Using single cell transcriptomic profiling of 48,000 high quality cells from early stage human embryos we find clusters that agree with the large embryonic lineages ectoderm, mesoderm, endoderm and extraembryonic. The latter markers were confirmed lineage identity by canonical markers such as SOX2, T/Brachyury, and GATA6. Pseudotime analysis determined that there exist bifurcating developmental trajectories from the epiblast to germ layers in which transcription factors EOMES, SOX17 and MESP1 are dynamically expressed to make critical lineage decisions. Enriched signaling networks such as WNT, TGF- β , FGF and NOTCH were also inferred to be active

during germ layer specification through the ligand receptor inference. The primitive streak and nascent mesoderm had localized key interactions including that of WNT3A-FZD7 and BMP4-BMPRI1A. Lineage specifically regulated regulons were identified through transcription factor analysis which includes GATA3, OTX2, and ZIC3, which sync signaling response and cell fate, and functional enrichment had developmental pathway enrichments of gastrulation, epithelial to mesenchymal transition and axon guidance. These integrations give a detailed signaling atlas of early human embryogenesis and determine novel communication hubs that may have developmental significance.

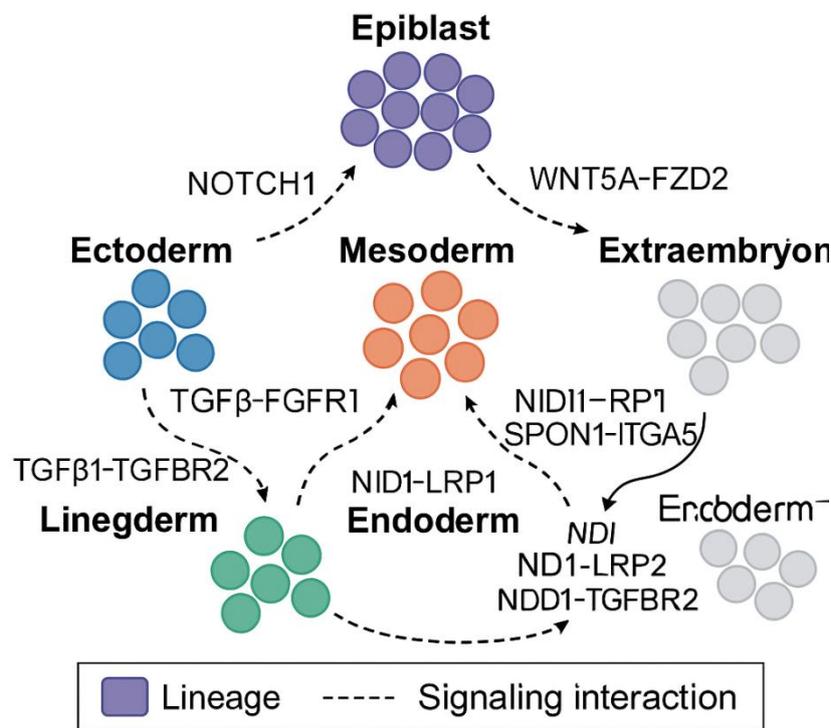


Fig 2. Predicted Lineage-Specific Signaling Interactions During Early Human Embryonic Development

Table 2. Novel Ligand–Receptor Pairs Identified in This Study

Ligand	Receptor	Source Cell Type	Target Cell Type	Biological Role
WNT5A	FZD2	Epiblast	Nascent mesoderm	Cell migration, EMT
BMP4	BMPRI1A	Extraembryonic ectoderm	Primitive streak	Mesoderm induction
TGF β 1	TGFBR2	Endoderm	Epiblast	Axis patterning
NID1	LRP1	Epiblast	Visceral endoderm	ECM remodeling
SPON1	ITGA5	Ectoderm	Mesodermal interface	Cell adhesion

5. CONCLUSION

To better understand these processes, this study presents a comprehensive study of the early human embryonic development, providing a detailed correlation of the interactions of molecular genes that define the development of lineage specification and morphogenesis. Through the incorporation of various cutting edge

computational techniques, including pseudotime trajectory modeling, ligand receptor communication inference, and transcription factor regulon analysis, we demonstrate the dynamic orchestration of key developmental signaling pathways including WNT, TGF β , FGF, BMP and NOTCH. They showed these pathways to drive the

germ layer formation, control cell-cell cross-talk, guide critical fate decisions, during gastrulation.

To dissect how such transcriptional programs and extracellular signaling cues cooperate to create cellular identities, the use of state of the art tool Monocle 3, CellChat, NicheNet and SCENIC helped provide a high resolution view. We found also well established regulators (example SOX17, T, FOXA2) and novel ligand – receptor interactions, NID1 – LRP1 and SPON1 – ITGA5 that appear to play distinct roles in human specific developmental contexts not shared with model organisms. The findings imply that such species specific regulatory circuits are responsible for unique aspects of human development.

This study goes further to characterize the static states and to discover how dynamic events unfolded over time to transition from pluripotent epiblast to the lineage bifurcation of specialist embryonic and extraembryonic origins. In addition, an additional regulatory atlas and signaling framework provide a rudimentary reference for developmental biology. The relevance of these insights is wide transnational, presenting value to studies of congenital diseases, tissue engineering, as well as regenerative therapies that use human stem cells. Alongside the design, they also provide a blueprint for the design of organoid models that better recapitulate in vivo human development.

Although all of these advances are very important, there are limits to them. Although transcriptomic data alone is used for our conclusions, these will need to be validated functionally. Future directions that build on this framework include interpolating the spatial transcriptomics, live cell lineage tracing and CRISPR perturbation assays against rapid proteomic correlations to experimentally validate predicted signal hierarchies and regulatory modules. A full expansion of the analysis to later Carnegie stages and inclusion of epigenomics and proteomics will expand our understanding.

Overall, this study not only sheds insight into the mechanistic sphere of early human embryogenesis

but also provides a base for future studies aimed at the developmental controls, modeling of disease and the next round of therapeutic innovation.

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